Glucose uptake in marine cyanobacteria: regulation, expression of the transporter and effects on the proteome and metabolome

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PhD Thesis

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TITULO: *Glucose uptake in marine cyanobacteria: regulation, expression of the transporter and effects on the proteome and metabolome*

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TÍTULO DE LA TESIS: Glucose uptake in marine cyanobacteria: regulation, expression of the transporter and effects on the proteome and metabolome

DOCTORANDO: José Ángel Moreno Cabezuelo

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El trabajo realizado por el doctorando José Ángel Moreno Cabezuelo se puede considerar de excelente. Durante el tiempo transcurrido desde su inicio (tres años y tres meses), ha realizado un estudio exhaustivo sobre los efectos de la adición de glucosa en la expresión del gen *glcH*, codificante de un transportador de glucosa de alta afinidad en cianobacterias marinas. Además, ha usado técnicas de proteómica y metabolómica de última generación para analizar el efecto de la glucosa sobre el metabolismo de tres estirpes de *Prochlorococcus* y tres de *Synechococcus*. Es de reseñar que este es uno de los primeros estudios en el que se han utilizado ambas técnicas en picocianobacterias marinas. Por otra parte, ha construido estirpes mutantes en dos residuos aminoacídicos esenciales del transportador GlcH. Y por último, ha conseguido sobreexpresar y purificar dicho transportador, con el objetivo de realizar estudios estructurales sobre el mismo en el futuro.

La formación de José Ángel Moreno Cabezuelo ha sido de muy alta calidad, en nuestra opinión, aprendiendo tanto técnicas de bioquímica (expresión y purificación de proteínas, proteómica) como de biología molecular (estudios de expresión génica, diseño y construcción de mutantes), lo que le permitirá abordar nuevos retos científicos en el futuro con gran capacidad. Además, ha realizado un considerable esfuerzo de lectura e integración de bibliografía para la escritura de su tesis doctoral, que se ha reflejado en un manuscrito de alta calidad, escrito íntegramente en inglés. Es de reseñar que su tesis opta a la mención internacional, al haber realizado una estancia de tres meses en el laboratorio de la Dra. Margarida Archer (Oeiras, Portugal), en el que aprendió las técnicas de optimización de expresión de proteínas de membrana, como el transportador sujeto de la tesis.

Durante el desarrollo de estos estudios, José Ángel Moreno Cabezuelo ha realizado diversas comunicaciones en forma de póster y charla en congresos nacionales e internacionales, y además ha publicado un primer artículo en la revista *PeerJ,* en el que se describen los resultados obtenidos sobre expresión génica:

Moreno-Cabezuelo JA, López-Lozano A, Díez J & García-Fernández JM (2019) Differential *glcH* expression in response to glucose and light in marine picocyanobacteria. *PeerJ* 6:e6248. doi 10.7717/peerj.6248.

Además, hay en preparación un segundo artículo, en el que se integrarán los resultados de proteómica y metabolómica. Los resultados obtenidos con los mutantes en residuos aminoacídicos esenciales de GlcH se presentarán en un manuscrito adicional.

Consideramos que el doctorando José Ángel Moreno Cabezuelo ha realizado una labor extraordinaria para obtener su tesis doctoral. Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 22 de abril de 2019

Firma de los directores

Jesús Díez Dapena

José Manuel García Fernández

ABSTRACT

Prochlorococcus and *Synechococcus* are the two photosynthetic organisms more abundant on Earth. Both have become a model in marine ecology and are responsible for a significant part of the global primary production. Previous results from our team demonstrated that *Prochlorococcus* can take up glucose, finding an increase in genes involved in its metabolization upon glucose addition. Later it was shown that the Pro1404/*glcH* gene encodes a biphasic glucose transporter in *Prochlorococcus* sp. strain SS120. Also, glucose uptake was detected in natural *Prochlorococcus* populations in the Atlantic Ocean. Given that this gene is present in all *Prochlorococcus* and *Synechococcus* strains, we decided to perform comparative experiments to assess the capabilities for glucose utilization in several representative strains from both genera, subjected to different glucose concentrations and also to darkness. To this goal, we used a double approach, proteomics and metabolomics, analyzing also the effect of darkness on the protein expression when glucose is available. Our working hypothesis is that mixotrophy confers an evolutive advantage to *Prochlorococcus* over other microorganisms which share the ecological niche (oligotrophic areas in the oceans).

The observed changes in *glcH* expression after addition of increasing concentrations of glucose indicate that *Prochlorococcus* and *Synechococcus* detect those concentrations, adapting the transporter expression accordingly. The decrease of *glcH* expression induced by darkness shows the active nature of glucose uptake in marine picocyanobacteria. This decrease is stronger in *Prochlorococcus* sp. strain MIT9313.

The metabolomic and proteomic results show that *Prochlorococcus* and *Synechococcus* take up and use glucose. The addition of 5 mM glucose led to a strong metabolic change towards general anabolic patterns in all studied strains.

Finally, the high affinity glucose transporter GlcH from *Prochlorococcus* sp. strain SS120 has been overexpressed and purified, with the goal of performing structural studies in the future. Besides, two mutants in essential amino acid residues of the glucose transporter GlcH from *Prochlorococcus* sp. strain SS120 have been constructed.

Resumen

Procholorococcus y *Synechococcus* son los dos organismos fotosintéticos más abundantes en la Tierra. Ambos se han convertido en modelo en ecología marina, y son responsables de una parte importante de la producción primaria global. Resultados previos en nuestro grupo demostraron que *Prochlorococcus* puede transportar glucosa, observando un aumento en la expresión de genes implicados en su metabolización tras la adición de glucosa. Posteriormente, se demostró que el gen Pro1404/*glcH* codifica un transportador de glucosa de cinética bifásica en *Prochlorococcus* sp. SS120. Además, se detectó transporte de glucosa en poblaciones naturales de *Prochlorococcus* en el Atlántico. Dado que este gen está presente en todas las estirpes de *Prochlorococcus* y *Synechococcus*, decidimos realizar experimentos comparativos que abordaran las capacidades de utilización de glucosa en varias estirpes representativas de ambos géneros, sometidas a diferentes concentraciones de glucosa y también a la oscuridad. Para ello utilizamos una doble aproximación proteómica y metabolómica, analizando además el efecto de la oscuridad sobre la expresión de proteínas cuando hay glucosa disponible. Nuestra hipótesis de trabajo es que la mixotrofía confiere una ventaja evolutiva a *Procholorococcus* frente a otros microorganismos con los que comparte el nicho ecológico (zonas oligotróficas de los océanos).

Los cambios observados en la expresión de *glcH* después de la adición de concentraciones crecientes de glucosa indican que *Prochlorococcus* y *Synechococcus* detectan estas concentraciones, adaptando la expresión del transportador en consecuencia. La disminución de la expresión de *glcH* inducida por la oscuridad muestra la naturaleza activa de la captación de glucosa en las picocianobacterias marinas. Esta disminución es más pronunciada en *Prochlorococcus* sp. MIT9313.

Los resultados de metabolómica y proteómica demuestran que *Prochlorococcus* y *Synechococcus* absorben y utilizan glucosa. La adición de 5 mM de glucosa condujo a un fuerte cambio metabólico hacia patrones anabólicos generales en todas las estirpes estudiadas.

Finalmente, el transportador de glucosa de alta afinidad GlcH de *Prochlorococcus* sp. SS120 ha sido sobreexpresado y purificado, con el fin de realizar estudios estructurales posteriormente. Además, se han construido por mutagénesis dirigida dos mutantes en residuos de aminoácidos esenciales del transportador de glucosa GlcH de *Prochlorococcus* sp. SS120.

“El mejor maestro en la vida es la experiencia.
Te cobra caro, pero explica bien”
Anónimo

“Lo que hacemos en la vida
tiene su eco en la eternidad”
Gladiator

A mis padres
A mi hermano

Agradezco profundamente a todas las personas que me han ayudado apoyado y acompañado durante este "camino" llamado tesis doctoral

En primer lugar, a mis directores José Manuel y Jesús por acogerme en su grupo, tratarme de la mejor manera, ser grandiosos compañeros de trabajo y antes que jefes, excelentes amigos y tener una calidad humana inmensurable, gracias por confiar en mí y darme la oportunidad de ser doctor, y poder llevar a cabo una carrera investigadora, siempre os estaré eternamente agradecidos.
La realización de la presente tesis es fruto de las orientaciones, sugerencias y estímulos de mis tutores, los cuales me han conducido durante estos años con talante abierto y generoso, guiándome y mostrando en cada momento una inmejorable disposición ante las dudas que durante la realización de la misma me surgieron, aportando valiosas observaciones que en todo momento guiaron esta investigación.

A todos mis compañeros de laboratorio, ya que sin su ayuda nada habría sido posible, pilares fundamentales en todo momento. Por la inmensa paciencia que Antonio ha tenido conmigo enseñándome gran parte de las técnicas y competencias que he adquirido y desarrollado en la tesis, mostrando siempre una sonrisa, buena disposición y resolución ante todos mis problemas y dudas, nunca tendré suficientes palabras de agradecimiento para darte las gracias, has sido para mi un excelente ejemplo de motivación, tesón y perseverancia, amante de la disciplina y de lo "bien hecho" gracias de nuevo. De la misma forma Tina, aunque no ha estado durante todo el desarrollo de mi tesis, aprendí muchísimo de ella, aunque a veces los protocolos me costaran contarle "cotilleos varios" y estoy seguro de que seguiré aprendiendo de ella cuando vuelva. A Mari por su buena disposición y atención, por entre muchas cosas, enseñarme a llevar a cabo estudios de transporte de glucosa, seguro que acabábamos los estudios con grandes resultados y por ser una gran compañera de la que espero seguir aprendiendo, gracias. A Lupe por enseñarme el maravilloso mundo de la proteómica, y ayudarme siempre con todo, ya sabes que como siempre te digo "Luupe, en mi casa en un altar" miles de gracias por todo.
A Yesi, la incorporación más reciente, por ayudarme siempre en lo que haga falta, y tener siempre "a los bebés" bien cuidados. A Mari Carmen por enseñarme a renovar, por tener siempre los medios preparados, todo limpito y curioso, por tener inmensa paciencia cuando llegaba con las "oleadas" de botes y por los litros y litros de medio cultivo que ha preparado, sin duda una excelente ayuda y pieza fundamental.
Como no a Juanito, gran compañero de laboratorio y amigo, por ayudarme en todo, así como enseñarme lo mejor y lo peor de Córdoba, y "por la improvisación y mejora de protocolos de laboratorio". A Juan Manuel, nuestro compañero doctorando de Ecuador mostrando siempre una buena disposición y ofreciendo su ayuda. A Alberto, aunque la mayoría de las veces metiera la pata, el chaval tenía buena intención y me ofrecía bastante ayuda. A los nuevos TFM, Manuel y Ramón por los buenos ratos.
A Goyi, otra persona que escriba lo que escriba nunca tendré suficientes palabras de agradecimiento para expresar todo lo que ha hecho por mí y me ha enseñado, con una nobleza digna de un rey y un corazón que no le coge en el pecho, me siento extremadamente orgulloso que formes parte de mi tribunal, gracias.
A Majo, mi compañera de tesis por todos los momentos compartidos, excelente amiga que me ha ayudado en todo.
A Toño por las buenas charlas y momentos, mostrando siempre grandes aportes y sugerencias que han sido de gran ayuda. A Alicia por darme el mejor trato desde que llegue, siempre con buenas palabras y ofreciéndome su ayuda con cualquier cosa que me hiciera falta, muchas gracias. A Emilia por su simpatía y ayuda, siempre te estaré

agradecido por pasarme alguna de tus prácticas y por esas tortillas que siempre desaparecen misteriosamente al cabo de un par de minutos de sacarlas. A Pepe Peinado, por enseñarme "truquillos" en el mundo de la enología y consejos para la vida en general, esta pendiente que hagamos el vino a ver que sale. A Pepe Alhama por buenos momentos entre risas, sobre todo gracias por haberme dejado sacarle partido a tu "ultra". A Jose Rafa por las grandes carcajadas y su peculiar "amor a Jaén". A Raquel por ayudarme y estar siempre con una sonrisa. A José Manuel Roldán por sus grandes palabras llenas de sabiduría y buenos momentos. A Oriol aunque haya pasado momentos puntuales con el, me ha dado grandes consejos y también aprender que "se sufre pero se gosa". A Rosa por ser una gran compañera y por todos lo buenos momentos que hemos pasado, espero que te vaya estupendamente en tu nueva etapa. A Dani el nuevo doctorando, siempre simpático y agradable. A Bea por los buenos ratos, espero que también te vaya genial en tu nuevo grupo. A Lidia por grandes momentos compartidos, se te echa de menos por aquí.

Durante la realización de mi tesis he tenido la suerte de realizar una estancia en Portugal en el laboratorio de Margarida Archer, donde me acogieron como uno más y me enseñaron con gran empeño técnicas de sobreexpresión de proteínas de membrana que tanto lío suelen dar. De manera especial agradecer a Diogo Athayde por estar conmigo en todo momento en el laboratorio, mostrando siempre una actitud y entrega máxima, sin duda no había tenido buenos resultados si no hubiera sido gracias a ti, siempre te estaré eternamente agradecido, espero volver pronto y que acabemos con la cristalización del transportador que tantos dolores de cabeza nos ha dado, también por los buenos momentos en la comida, los vídeos de risa en el laboratorio y por enseñarme un "portuñol" de calidad *muito obrigado*. Y como no al resto del grupo que siempre han estado atentos para cualquier cosa que me hiciera falta Margarida, José Rodrigues, José Brito, Márcia y Paulo.
Sin olvidar a mis compañeros de piso durante la estancia "Rafman" y Pierre que me hicieron pasar unos meses estupendos, y a mi casera Inês por tratarme como si fuera su hijo.

También por los cientos de celebraciones en el grupo; perol de los pepes, comida de navidad, cena de verano, celebraciones de artículos, cumples, proyectos, becas, perol de los Pepes entre otras, el café de las 10.30... Son tantos momentos y entre buen ambiente, que hace que el venir a trabajar sea un auténtico y puro disfrute.

A los amigos de siempre: Dani, Rubén, Juanru, Jaime, Juanma, Alex y Juan por todos los momentos de risas, comidas, salidas, viajes y "festejos varios" gracias por estar siempre ahí y haced que todo sea más llevadero.

A todos los miembros del C-6 por su ayuda, entre ellos a Pedro Piedras por hacerme siempre reír y por sus temas de conversación interesantes. A Alejandro Chamizo por su ayuda con el sonicador y la sobreexpresión de proteínas, a Victoria por su ayuda con cualquier tema.
A las secretarias por ser simpáticas y ayudar siempre que cualquier tema administrativo.
A Pilar de recepción por saludar siempre con una sonrisa.
A Mercedes de genómica por las buenas secuenciaciones, aunque a veces costara.
A María Dolores por ayudarme siempre con las prácticas y tenerlo siempre preparado todo perfecto.

A Carlos del servicio de proteómica por buscarnos “hueco rápido” para correr las muestras y sus consejos y ayuda en el aprendizaje de proteómica.

A José Antonio Herrera Cervera por ser mi tutor de trabajo de fin de licenciatura y despertar en mi el “gusanillo de la ciencia”.
A María Jesús Delgado, y Mª Jesús Torres mis tutoras del TFM por permitirme un gran trabajo con vosotras, el cual me ha permitido llevar a cabo este doctorado.

A mis preparadores a nivel deportivo Diego Villegas y Xisco Serra, por enfocarme en los valores de disciplina, esfuerzo, superación, perseverancia, entre otros muchos y extrapolarlos a mi vida diaria para ser mejor en todos los aspectos.

A los proyectos y entidades financiadoras que han permitido que haya podido llevar a cabo la tesis doctoral BFU2016-76227-P (MINECO), P12-BIO-2141 (Junta de Andalucía) & Programa Propio de Investigación (Universidad de Córdoba).

También me gustaría agradecer a todos los componentes que han formado en el pasado parte del grupo BIO-123, por sus aportaciones y conocimientos al mismo, y aquellos miembros, que aunque ya no se encuentren entre nosotros, están siempre presentes. De manera especial a Fermín, una gran persona a la que tuve suerte de conocer, descanse en paz.

Por último, a mi familia, por su empuje y apoyo incondicional, sois los cimientos de mi desarrollo, en el diccionario no hay palabras para daros las gracias. Siempre me aconsejasteis y mostrasteis el mejor camino a seguir, gracias por los valores que me habéis transmitido. A mi padre por ser mi guía y por empujarme cuando tenía miedo de seguir mis sueños, sé que te sentirás bien orgulloso allá donde estés.

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aa	aminoacid
Amp^r	ampicillin resistance
APS	ammonium persulfate
ASW	artificial seawater
atm	atmosphere
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
^{o}C	centigrade
C_t	cycle treshold
cDNA	complementary DNA
DON	dissolved organic nitrogen
Da	Dalton
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
DNA	deoxyribonucleic acid
DBMIB	2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
CoA	Coenzyme A
DCMU	diuron, 3-(3-4-dichlorophenyl)-1,1-dimethylurea
DDM	n-Dodecyl beta-D-maltoside
DM	Decyl β-D-maltopyranoside
EDTA	ethylene-diamine-tetraacetic acid
µE	µEinstein
FDR	false discovery rate
GDH	glutamate dehydrogenase
GS	glutamine synthetase
GOGAT	glutamate synthase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropil-β-D-1-thiogalactopyranoside
K_m	Michaelis-Menten constant
K_m^r	Kanamycin resistance

LB	Luria-Bertani
Mb	megabase
NADPH	nicotinamide adenine dinucleotide phosphate
Ni-NTA	nickel-nitrilotriacetic acid
^{o}N	north
NADH	nicotinamide adenine dinucleotide
PON	Particulate organic
Psi	pounds-force per square inch (1 atm = 14,6956 psi)
PSI	photosystem I
PSII	photosystem II
PCC	Pasteur Culture Collection
PE	phycoerythrin
PCR	polymerase chain reaction
RNA	ribonucleic acid
^{o}S	south
SDS	sodium dodecyl sulphate
SD	standard deviation
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
qRT-PCR	reverse transcription polymerase chain reaction
TCA cycle	tricarboxilic acid cycle
TEMED	N,N,N´,N´-Tetramethylethylenediamine
TFA	trifluoreacetic acid
UV	ultraviolet
PCA	Principal component analysis
T_m	melting temperature
Tween 20	Polysorbate 20

INTRODUCTION

Introduction

1. THE OCEAN

The ocean is the largest habitat on Earth, it represents approximately 70% of the Earth's surface. The sea surface and seabed areas over 350 million km^2, the ocean volume exceeds 1.3 billion Km^3 (or 1.3 sextillion liters) (Costello et al., 2010). Most of the ocean is dark, cold and deep, although it is important to realize that there are some zones containing an abundance of ocean life because sunlight is available for photosynthesis.

1.1 Geophysics structure

The oceans are composed by two large areas; the pelagic zone (the lowest level of the ocean, including the sediment surface and subsurface) and the benthic zone (the water column in the open ocean):

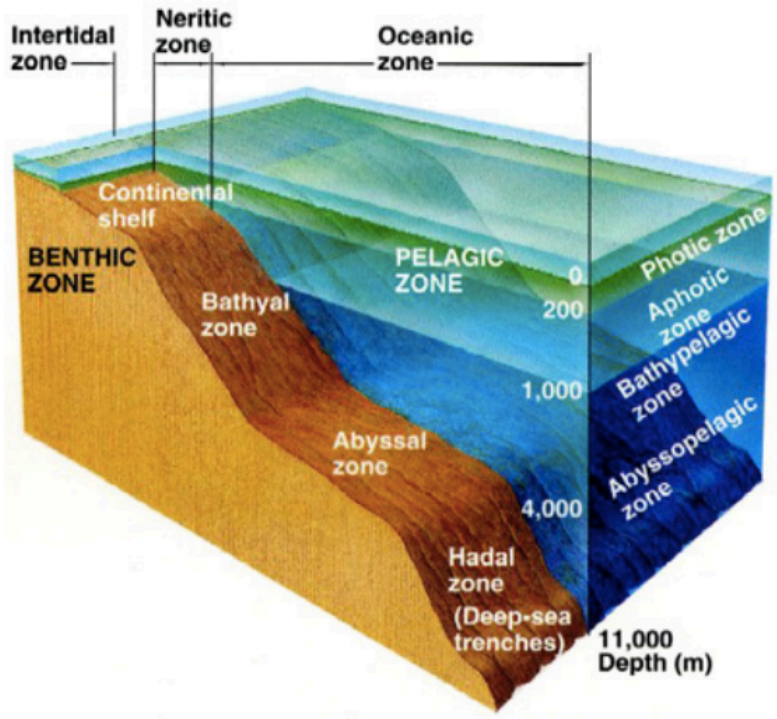


Figure 1. Areas of the ocean. (Image taken from http://marinebio.org/oceans/deep/)

Benthic–pelagic coupling is manifested as the exchange of mass, energy and nutrients between benthic and pelagic habitats. It has an important role in aquatic ecosystems, and is essential for many functions, such as the nutrient cycle and energy transfer in food webs (Griffiths et al., 2017).

Pelagic zone refers to the open water in which swimming and floating organisms live. Organisms living there are called the pelagos. From the shallowest to the deepest, biologists divide the pelagic into the epipelagic (less than 200 m, where there can be photosynthesis), the mesopelagic (200 - 1,000 m, the "twilight" zone with faint sunlight but no photosynthesis), the bathypelagic (1,000 - 4,000 m), the abyssopelagic (4,000 - 6,000 m) and the deepest, the hadopelagic (the deep trenches below 6,000 m to about 11,000 m). The last three zones have no sunlight at all.

The traditional view of benthic–pelagic coupling has focused on the deposition of nonliving organic material to benthic habitats (Hargrave, 1973), bioresuspension, (Graf and Rosenberg, 1997) and the release of inorganic nutrients from the sediments. These fluxes have been quantified in a multitude of ecosystems (Duineveld et al., 2000; Smith et al., 2016). However, benthic–pelagic coupling has been defined as those processes which connect the bottom substrate and the water column habitats through the exchange of mass, energy, and nutrients (Griffiths et al., 2017).

1.2 Light in the oceans

The radiations that form the light are absorbed by the sea water and transmit heat to it. This absorption is selective and depends on the wavelength of the radiation. The light that penetrates the ocean is indispensable for the development of marine life (García-Pichel, 1998).

Photosynthetic organisms in the ocean such as algae and phytoplankton must live in well-lit surface waters called the euphotic zone. The euphotic zone, photic zone or sunlight zone is the upper part of the ocean that receives bright and clear sunlight.

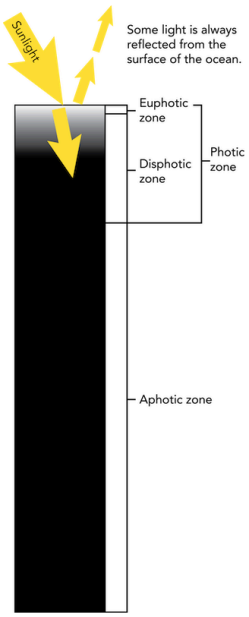


Figure 2. Solar radiation in the ocean. (Image taken from https://manoa.hawaii.edu/)

In clear tropical waters, the euphotic zone may extend to a depth of 80 m. Sunlight does not penetrate as deeply near the poles, so in these areas the euphotic zone may be less than 10 m deep. Turbid, muddy waters may have a euphotic zone only a few centimeters in depth. The disphotic zone is the water layer beneath the euphotic zone (figure 2). In clear, open ocean ecosystems, the euphotic zone can extend to 200 m (Karl and Church, 2014). The photic zone is made up of the euphotic and disphotic zones. The aphotic zone is the water layer where there is no visible sunlight. Most of the water in the ocean lies in the aphotic zone. Some lakes are also deep enough to have aphotic zones.

1.3 Ocean temperature

The temperature is a determining factor of the ecological distribution of the species that inhabit the ocean. As the depth increases there is less radiation, so the temperature decreases, forming a layer of hot water on the surface, with a uniform temperature that can range from 20 to 500 m deep (Cifuentes-Lemus et al., 2003).

Following this zone there is a boundary zone from which the temperature drops rapidly; this bordering area is the thermocline, which separates these surface waters, less dense and less saline, from the deeper, colder, denser and saline waters (McLellan, 1977).

It is convenient to divide the oceanic column into three regions when discussing its temperature:

-The surface layer is usually somewhat less than 200 m thick and is that portion of the column which experiences seasonal changes in response to the exchange of energy with the atmosphere and the absorption of solar radiation.

-The permanent thermocline is below the surface layer and that region is where, at the height of winter, the most rapid decrease in temperature occurs with depth.

-The deep water below the permanent thermocline often shows a gradual decrease in temperature with depth to the bottom.

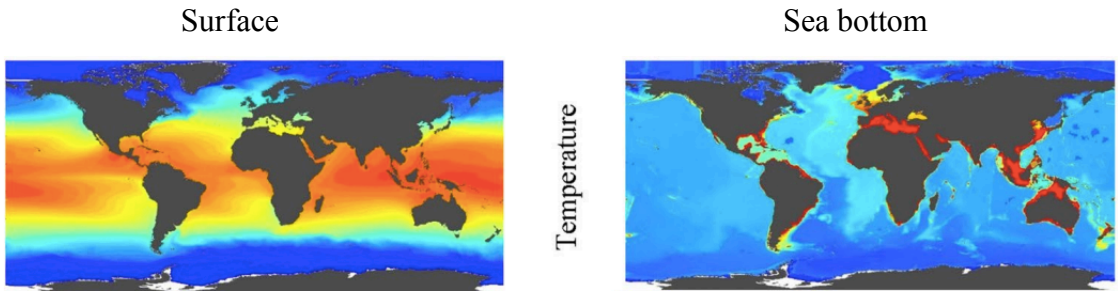


Figure 3. Contrast of sea surface (left) and sea bottom (right) values of environmental temperatures. The GMED (Global Marine Environmental Datasets) colour scale goes from red (high) to blue (low). Figure taken from (Costello et al., 2018).

Most variables differ strikingly between the surface and sea bottom (figure 3). The primary plant nutrients, nitrogen, phosphate and silicate are much higher near the seabed than on the sea surface, but temperature is lower.

1.4 Distribution of nutrients in the ocean

In the euphotic zone of the oceans, nutrients are used by phytoplankton and cyanobacteria. The respiration and decomposition of marine organisms cause the nutrients to be released again in seawater, mainly in deep waters. As a result, nutrient conditions are generally low on the surface of deep water.

The main nutrients for the correct development of the marine plankton are nitrogen, phosphorus, and iron, however the concentration of each element is different in the ocean. Many years ago Redfield recognized that the dissolved nutrient ratios in the deep are corrected remarkably to the ratios of particulate organic matter in surface waters (Redfield, 1934). He assigned this correlation to the fact that organic matter (phytoplankton biomass) is produced, on average, in a ratio that is determined by biochemical needs, and that this material is subsequently remineralized in the deep ocean, having a release of inorganic nutrients in the same proportions (Sohm et al., 2011). This ratio is 106 C : 16 N : 1 P, and it links three major biogeochemical cycles through the activities of marine phytoplankton. However, some studies show that the relation of Redfield is not universal, but represents the average term of carbon to nitrogen ratio (Klausmeier et al., 2004; Arrigo, 2005).

Carbon and nitrogen are essential, although their abundance and concentration are very different; the carbon is found mainly in the lithosphere, with less than 0.05 % being cycled through the atmosphere, biosphere and hydrosphere.

Inorganic nitrogen exists in a variety of molecular forms, phases and redox states (Capone, 2000), although nitrogen is accepted as the nutrient limiting primary production of phytoplankton. Most of it is in gaseous form in the atmosphere, and is fixed by physical processes, such as the formation of nitrogen oxide, or biological processes (Capone et al., 2006).

Table 1. Concentrations of inorganic nitrogen species in the ocean (Capone, 2000).

Form	Oceanic concentrations (μM)	Coastal concentrations (μM)	Global marine pools (Tg)
GASEOUS			
Dinitrogen (N_2)	900-1100	900-1100	22-23 x 10^3
Nitrous oxide (N_2O)	0.006-0.07	0-0.25	0.2-0.8
Nitric oxide (NO)	?	?	?
INORGANIC			
Nitrate (NO_3^-)	<0.03- >40	<0.1-200	570-677
Nitrite (NO_2^-)	<0.03-0.1	<0.03-10	?
Ammonium (NH_4^+)	<0.03-1	<0.03->100	7-8
ORGANIC			
Dissolved organic (DON)	3-7	3-20	63-530
Particulate organic (PON)	0.07-0-5	0.1-30	3-24
Amino acids	0.15-1.5	0.12-6.6	

The largest nitrogen reservoir in the oceans is dissolved N_2, which is found in concentrations of almost 1 mM (Sharp, 1983; Capone, 2000). NO_3^- has a common distribution, with low concentrations in the euphotic zone (<0.03-10 μM) and high in the aphotic zone (30-40 μM). However, in tropical and subtropical zones, NO_3^- is at a concentration below 10 nM, which suggests that NO_3^- can limit the growth of plankton (Nagata, 2008). The dissolved organic nitrogen (DON) is found in concentrations in the ocean between 3-7 μM. Dissolved organic nitrogen has an important role: compounds such as amino acids and urea are part of this group (Eppley and Peterson, 1979; Sohm et al., 2011).

The fixed nitrogen (N) limits productivity across much of the low-latitude ocean (Landolfi et al., 2018).

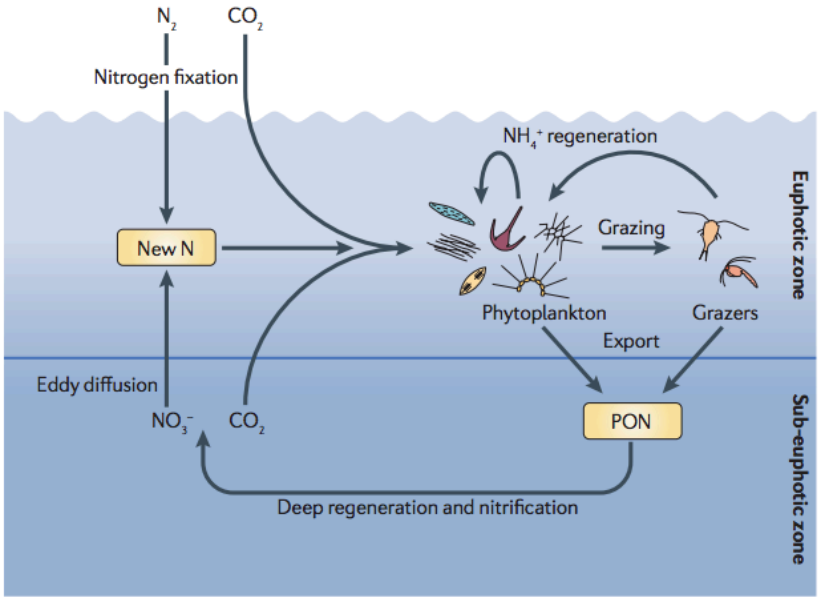


Figure 4. Importance of nitrogen fixation in the ocean. Image taken from (Sohm et al., 2011).

The exchange of organic material between surface and deep waters depends on the amount of CO_2 fixation (primary production), which in turn depends on new nitrogen inputs (N_2 fixation or nitrate diffusion from deep waters) (figure 4) (Sohm et al., 2011).

The N cycle is an important component of the biogeochemical cycles of the water column of the ocean (figure 5). N is in short supply relative to other nutrients needed for growth and, therefore, is the most limiting nutrient (Zehr and Kudela, 2011).

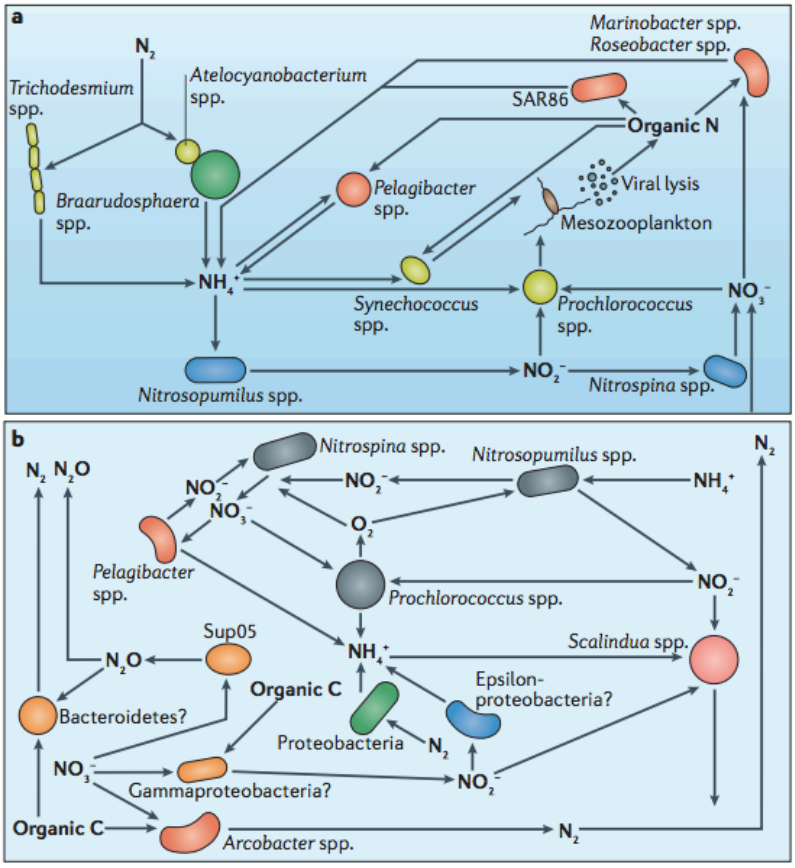


Figure 5. Potential nitrogen-transforming microbial networks in different ecosystems (Kuypers et al., 2018). Figure 5a. In surface waters of the ocean that receive sunlight, cyanobacteria mainly assimilate ammonium and organic nitrogen compounds. Nitrogen-fixing bacteria are responsible for providing an extra amount of ammonium. As the depth in the ocean increases, the oxidation of ammonium to nitrate is carried out. The majority of the nitrate produced is transferred to surface water and will later be assimilated by phytoplankton. In figure 5b. There are waters rich in nutrients that stimulate primary production in surface waters due to the action of the wind. There is a great loss of nitrogen in the minimum oxygen zones by the action of anaerobic ammonium-oxidizing bacteria and in a lower percentage, denitrification.

Phytoplankton normally prefers ammonium to nitrate, since reduced nitrogen requires less metabolic energy for assimilation (García-Fernández and Diez, 2004; García-Fernández et al., 2004; Flores and Herrero, 2005; Luque et al., 2008). Recent studies have shown that ammonium is subjected to rapid recycling in the ocean, facilitating an efficient transfer of ammonium in plankton communities (Klawonn et al., 2019). Nutrient recycling has been demonstrated to facilitate the long-term stability of interactions between marine phototrophic and heterotrophic organisms (Christie-Oleza et al., 2017).

Nitrogen fixation is carried out by the action of several types of diazotrophs. For instance, *Trichodesmium* spp. is a type of marine cyanobacterium that has the ability to

fix nitrogen and take up nitrogen at the same time. The N_2 fixation and the diversity of diazotroph species in the < 10 μm plankton was recently discovered (Zehr et al., 2001; Zehr et al., 2017).

The availability of inorganic nitrogen fixed in its various forms (nitrate, nitrite and ammonium) largely limits the primary productivity in the ocean.

In the ocean there are large variations of ammonium and nitrate fluxes (Zehr and Ward, 2002). For example, the oligotrophic waters of the subtropical gyres cover more than 60% of the total surface of the ocean and contributes to more than 30% of the carbon fixation in the sea. In that area, the concentration of NO_3^- was undetectable (Marañón et al., 2003; Marañon, 2005).

It has been shown that nitrogen is the most limiting nutrient in the ocean. However, it has also been described that many areas of the ocean have limitations in other compounds such as phosphorus and iron (Martin, 1992; Sohm et al., 2011), in the upper mixed layer of the oligotrophic regions, the phosphate concentration was between 0.01-0.02 μM. Moreover, in the subtropical gyre of the North Pacific, an average concentration of 50 nM phosphorus was reported (Bjorkman et al., 2000). The iron in the ocean is the result of dust deposition from nearby desert areas; it should be noted that the oceans of the southern hemisphere receive lower dust inputs than the northern oceans (table 2) (Sohm et al., 2011).

Table 2. Phosphorous and iron concentration in different oceans (Sohm et al., 2011).

Location	Dust deposition	Fe (nM)	DIP* (nM)
North Atlantic Ocean	Very high	0.2-1.2	0.2-5
South Atlantic Ocean	Very low	0.1-0.4	ca. 200
North Pacific Ocean	Low	0.1-0.3	10-100
South Pacific Ocean	Very low	0.1-0.2	110-240
Arabian Sea	High	0.5-1	BD*-1,000
Mediterranean Sea	High	0.2-1.2	BD-70
Baltic Sea	Low	3-7	BD-800

*BD: below detectable limits, *DIP: dissolved inorganic phosphorous.

2. CYANOBACTERIA

An important role of cyanobacteria is the original oxygenation of the atmosphere about 2.4 billion years ago (Capone, 2001; Falkowski and Oliver, 2007). However, the marine cyanobacteria appeared much later about 600 millions years ago (Sanchez-Baracaldo, 2015).

Cyanobacteria are among the most abundant photosynthetic organisms in the oceans (Roitman et al., 2018). These oxygenic photoautotrophic prokaryotes are one of the most important lineages of the domain *Bacteria* (Waterbury and Rippka, 1989; Giovannoni and Rappé, 2000). There are two types, α- and β-cyanobacteria (Badger et al., 2002), the classification is based on the content of carboxysomes and rubisco. The α- are formed by the marine cyanobacteria *Prochlorococcus* and *Synechococcus* (Ohashi et al., 2011).

Cyanobacteria are very important in the ocean, but especially in tropical ecosystems where they are very numerous and diverse (Partensky et al., 1999; Scanlan and West, 2002; Scanlan, 2003; Flombaum et al., 2013). *Prochlorococcus* and *Synechococcus* are the dominant primary producers in ocean ecosystems and perform a significant fraction of ocean carbon fixation.

2.1 Marine picocyanobacteria

Cyanobacteria, as primary producers, play a very important role in the carbon cycle of the Earth and also intervene in the nitrogen cycle. Therefore, these organisms are of great importance for the maintenance of the biosphere (Partensky et al., 1999; García-Pichel et al., 2003; Heywood et al., 2006; Mella-Flores et al., 2012).

Marine picocyanobacteria are the most abundant organism on Earth, with only two genera, *Prochlorococcus* (Chisholm et al., 1988; Partensky et al., 1999; Heywood et al., 2006; Johnson et al., 2006; Partensky and Garczarek, 2010; Biller et al., 2014; Chisholm, 2017) and *Synechococcus* (Waterbury et al., 1979; Scanlan and West, 2002; Scanlan, 2003; Dvorak et al., 2014) dominating most of the oceans.

Prochlorococcus and *Synechococcus* are thought to have a common ancestor about 150 million years ago (Waterbury et al., 1979; Dufresne et al., 2005; Dufresne et al., 2008) and occupy complementary niches in the ocean. Although other studies have reported a different estimate (1,000-542 million years ago) (Sanchez-Baracaldo, 2015).

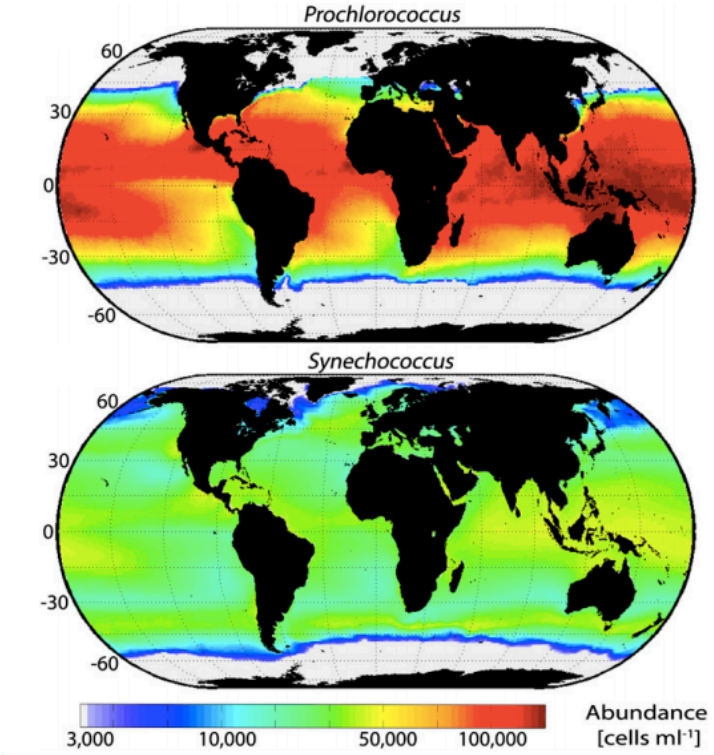


Figure 6. Present global distribution of *Prochlorococcus* and *Synechococcus* abundance. (A) *Prochlorococcus* and (B) *Synechococcus* mean annual abundances at the sea surface (Flombaum et al., 2013).

2.1.2 *Prochlorococcus*

Prochlorococcus is the most abundant photosynthetic organism on the planet. The estimation of *Prochlorococcus* cells in the oceans is 3 x 10^{27} (Chisholm, 2017). *Prochlorococcus* is the numerically dominant phototroph in oligotrophic subtropical gyres, which are among the largest contiguous biomes on Earth (Karl, 1999), this cyanobacterium was discovered in 1988 (Chisholm et al., 1988). *Prochlorococcus*

constitute half of the chlorophyll in ocean ecosystems, supplying by itself significant amounts of organic carbon to the rest of the microbial food web (Chisholm, 2017), and is one of the most studied marine organisms, since it has great ecological importance. Due to advances in genomics and metagenomics, many genomes have been sequenced, which provide a lot of data about metabolic diversity (Dufresne et al., 2003; Rocap et al., 2003; Farrant et al., 2016; Berube et al., 2018; Biller et al., 2018).

Size and structure

Prochlorococcus is the smallest known photosynthetic organism (Partensky et al., 1999). The size estimate is about 0.5 to 0.8 µm for length and 0.4 to 0.6 µm for width. *Prochlorococcus* contains in its cytoplasm DNA filaments, carboxysomes and glycogen granules (figure 7), located in the proximity of the thylakoids or directly between them (Lichtle et al., 1995).

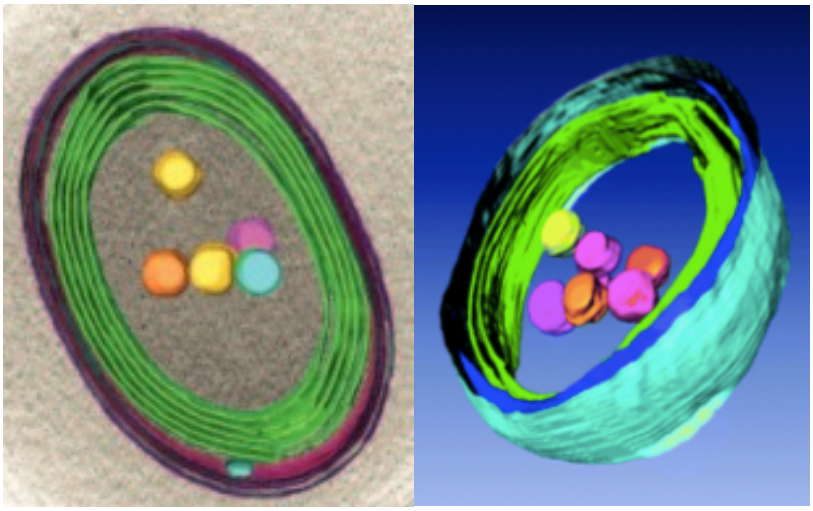


Figure 7. Cellular model of *Prochlorococcus* MIT9313. Images were obtained by cryoelectron tomography. Structures incorporate the cell wall (purple, pink, blue), carboxysomes (yellow, pink, orange, blue) and extensive intracytoplasmic membrane system (green) (Ting et al., 2007).

Photosynthetic apparatus

The strategy of light-harvesting of *Prochlorococcus* differs from that of other cyanobacteria, which use phycobilisomes for this function (Tooley and Glazer, 2002).

Prochlorococcus has a much simpler pigmentation specifically adapted to collect the blue light at depth in the sea. *Prochlorococcus* has replaced the phycobilisomes by the proteins encoded by the *pcb* genes. These proteins bind chlorophylls a_2 / b_2 serving as antenna proteins (Chisholm et al., 1992; Goericke and Repeta, 1992, 1993; Partensky and Garczarek, 2003; Steglich et al., 2003), with the advantage of being much less bulky than phycobilisomes (La Roche et al., 1996). Moreover, *Prochlorococcus* uses monovynil chlorophyll *b* as an accessory pigment in the antenna complex (Moore et al., 1995; Ting et al., 2002; Scanlan et al., 2009). These unique pigments increase the absorption of blue light by *Prochlorococcus* (Chisholm et al., 1988; Goericke and Repeta, 1992).

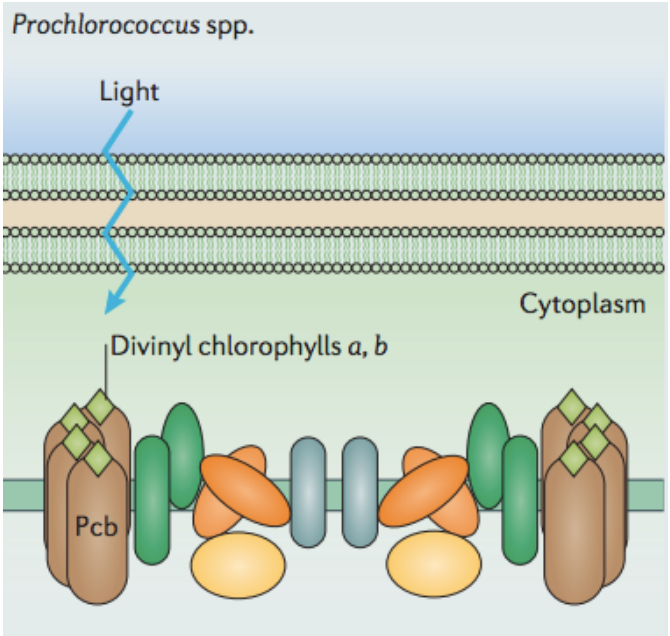


Figure 8. Scheme of the photosynthetic apparatus of *Prochlorococcus*. Image taken from (Biller et al., 2015).

In all strains of *Prochlorococcus* there is a lycopene cyclase, which is responsible for the synthesis of α-carotene (Hess et al., 2001) although it is found in low concentration in the cell (Partensky et al., 1993; Moore et al., 1995) therefore, in *Prochlorococcus* this

pigment must be important for the correct functioning of the photosynthetic apparatus (Partensky and Garczarek, 2010).

Oceanic distribution

The abundance of *Prochlorococcus* ecotypes in different ocean regions has been studied extensively for a long time (Scanlan and West, 1999; Bouman et al., 2006; Zinser et al., 2006; Zinser et al., 2007; Scanlan et al., 2009; Malmstrom et al., 2010; Farrant et al., 2016; Braakman et al., 2017; Kent et al., 2019). *Prochlorococcus* is distributed mainly in oligotrophic areas between 45º North and 45º South (Olson et al., 1990; Johnson et al., 2006). *Prochlorococcus* has a very wide oceanic distribution. This distribution indicates that low temperatures are lethal for *Prochlorococcus*, however, *Synechococcus* resists temperatures much lower than *Prochlorococcus* (Shapiro and Haugen, 1988; Pittera et al., 2014). *Prochlorococcus* is found in marine waters, with a temperature between 26-29 ºC (Malmstrom et al., 2010).

The highest concentration of *Prochlorococcus* cells is found between 100-200 m depth (Chisholm et al., 1988; Jameson et al., 2008; Zwirglmaier et al., 2008).

Prochlorococcus ecotypes have geographically and seasonally dynamic populations that in warm, stable stratified water columns always return to the same structure (Bouman et al., 2006; Johnson et al., 2006; Malmstrom et al., 2010; Larkin et al., 2016).

Recently diverging highlight-adapted (HL) ecotypes are most abundant in the surface, whereas deeper branching low-light-adapted (LL) ecotypes are most abundant at depth (Moore et al., 1998; Scanlan and Wilson, 1999) (figure 9).

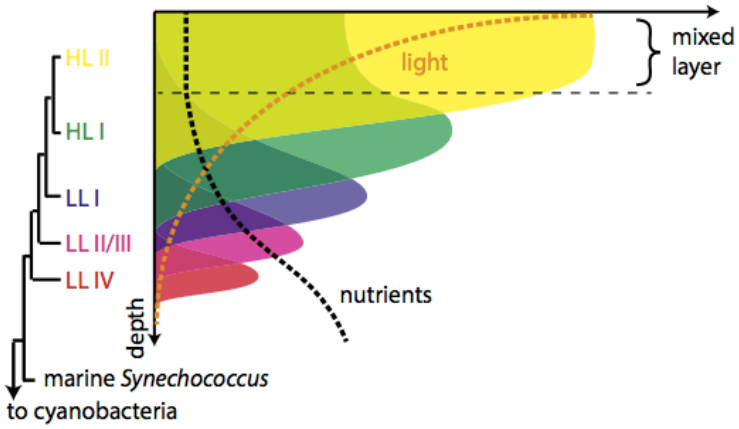


Figure 9. Relative abundance distributions of *Prochlorococcus* ecotypes. As a function of depth and accompanying light intensity and nutrient concentration profiles in stratified oceanic waters (Braakman et al., 2017).

The ecotypes of surface (HL, high light-irradiance adapted), are the most abundant in the high regions of the euphotic zone, however, the ecotypes of depth (LL, low light / irradiance adapted), are the most abundant strains in the low regions of the euphotic zone. They live in an area where the concentration of nutrients is high and there is little light, they are also the oldest evolutionarily (Moore et al., 1998; West and Scanlan, 1999; Bouman et al., 2006; Johnson et al., 2006; Malmstrom et al., 2010).

Depending on the clade, the ecological distribution of largest abundance will be different (table 3):

Table 3. Ecological characteristics of the areas where the different clades of *Prochlorococcus* are more abundant, modified from (Scanlan et al., 2009).

Clade	Ecological distribution of largest abundance
HLI	Between 35º and 48º N and 35º and 40º S, weakly stratified surface waters.
HLII	Subtropical and tropical regions between 30º N and 30º S, strongly stratified surface waters.
LLI	This clade occupies an "intermediate" position in the water column in stratified waters, in high latitude, can be found throughout the euphotic zone up to the surface.
LLIV	Abundantly distributed between 40º N and 35º S latitudinal range, but restrictive to deep euphotic zone.
LLII &LLIII	Are present in deep oceanic water at low concentrations.

Genomic characteristics

Prochlorococcus is a prime example of an organisms with "streamlined" genome (Giovannoni et al., 2014). An evolutionary strategy of *Prochlorococcus*, has been the genomic compaction, since it has eliminated non-coding zones (Dufresne et al., 2003; Rocap et al., 2003; Dufresne et al., 2005; Kettler et al., 2007; Dufresne et al., 2008; Holtzendorff et al., 2008; Partensky and Garczarek, 2010).

It has been demonstrated that *Prochlorococcus* possesses the smallest genome of all prokaryotes evolving oxygen. Most strains of *Prochlorococcus* have a low content in G+C (Watson and Tabita, 1996) (table 4).

Table 4. Most representative *Prochlorococcus* genomes (Biller et al., 2014).

Prochlorococcus	Subcluster/ Ecotype	Clade no.[a]	Genome size	GC (%)
MED4	HL	HLI	1.66	31
MIT9515	HL	HLI	1.7	31
MIT9301	HL	HLII	1.64	31
AS9601	HL	HLII	1.67	31
MIT9215	HL	HLII	1.74	31
MIT9312	HL	HLII	1.71	31
NATL1A	LL	LLI	1.86	35
NATL2A	LL	LLI	1.84	35
SS120	LL	LLII	1.75	36
MIT9211	LL	LLIII	1.69	38
MIT9303	LL	LLIV	2.68	50
MIT9313	LL	LLIV	2.41	51
EQPAC	HL	HLI	1.65	30.8
GP2	HL	HLII	1.62	31.2
MIT0604	HL	HLII	1.78	31.2
MIT9107	HL	HLII	1.69	31
MIT9116	HL	HLII	1.68	31
MIT9123	HL	HLII	1.69	31
MIT9201	HL	HLII	1.67	31.3
MIT9302	HL	HLII	1.74	31.1
MIT9311	HL	HLII	1.71	31.2
MIT9314	HL	HLII	1.69	31.2
MIT9321	HL	HLII	1.65	31.2
MIT9322	HL	HLII	1.65	31.2
MIT9401	HL	HLII	1.66	31.2
SB	HL	HLII	1.66	31.5
MIT0801	LL	LLI	1.93	34.9
PAC1	LL	LLI	1.84	35.1
LG	LL	LLII, III	1.75	36.4
MIT0601	LL	LLII, III	1.7	37
MIT0602	LL	LLII, III	1.75	36.3
SS2	LL	LLII, III	1.75	36.4
SS35	LL	LLII, III	1.75	36.4
SS51	LL	LLII, III	1.74	36.4
SS52	LL	LLII, III	1.75	36.4
MIT0701	LL	LLIV	2.59	50.6
MIT0702	LL	LLIV	2.58	50.6
MIT0703	LL	LLIV	2.57	50.6
UH18301	HL	HLII	1.65	31.2
MIT9202	HL	HLII	1.69	31.1

[a] Ecotype as defined in reference (Kettler et al., 2007).

The genomes are composed of a single circular chromosome with no plasmids.

Nutrient assimilation

Prochlorococcus is able to grow in a wide range of irradiances in the waters of the ocean, and has a great ability to colonize oligotrophic areas (Partensky et al., 1999). Nutrients such as nitrogen and phosphorus are limiting in some oceanic waters, it has been proven that *Prochlorococcus* is able to colonize areas with extremely low phosphorus concentrations (Parpais et al., 1996; Zubkov et al., 2007; Krumhardt et al., 2013; Casey et al., 2016). Another important element in the ocean that limits primary production in remote areas of the oceans is iron (La Roche et al., 1995; Mills et al., 2004). The low iron requirement of *Prochlorococcus* could be a key factor in its success in central oceanic areas (Thompson et al., 2011).

2.1.3 *Synechococcus*

The marine cyanobacteria of the genus *Synechococcus* are important primary producers, due to displaying a wide latitudinal distribution that is underpinned by diversification into temperature ecotypes, and exhibit a unique diversity of photosynthetic pigments, allowing them to exploit a wide range of light niches (Pittera et al., 2017; Grebert et al., 2018; Pittera et al., 2018).

Synechococcus was detected earlier than *Prochlorococcus* due to their intense orange phycoerythrin fluorescence (Waterbury et al., 1979). *Synechococcus* cells are the dominant phycobilisome containing cyanobacteria in the oceans (Ting et al., 2002).

Marine *Synechococcus* is a complex taxon and their criteria of classification are based on their major light-harvesting accessory pigment profiles, their ability to carry out swimming motility and growth requirements (Waterbury et al., 1986; Urbach et al., 1998; Scanlan and West, 2002; Six et al., 2007a; Scanlan et al., 2009). Recent studies have used genes with high levels of diversity to perform refined classifications which show the global distribution of marine picocyanobacteria in great detail (Farrant et al., 2016; Grebert et al., 2018; Kent et al., 2019).

Size and structure

Marine *Synechococcus* is a coccoid to rod-shaped and range in size from 0.7-0.9 µm in diameter and 1-2.5 µm in length (Waterbury et al., 1985). The division is carried out by means of binary fission into equal halves in one plane, *Synechococcus* is characterized by photosynthetic thylakoid membranes located in peripherally and lack of structure sheaths (Waterbury and Rippka, 1989; Scanlan, 2003).

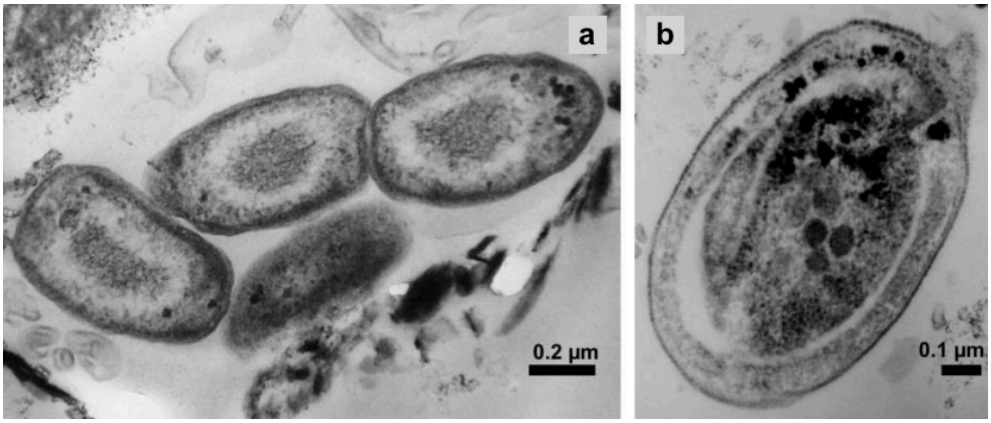


Figure 9. a, Three *Synechococcus* cells (0.72 x 0.41 µm). b, Detail of one *Synechococcus* cell (0.98 x 0.73 µm). Image taken from (Aquino-Cruz et al., 2013).

Oceanic distribution

Synechococcus is more abundant in nutrient-rich than in oligotrophic areas and its distribution is generally restricted to the upper well-lit layer. This cyanobacterium is present in most ocean waters in concentrations of $7.0 \pm 0.3 \times 10^{26}$ cells (Flombaum et al., 2013). *Synechococcus* is a major contributor to global oceanic primary production (Grebert et al., 2018) and performs a significant fraction of ocean carbon fixation (Li, 1994; Berube et al., 2018).

Table 5. Ecological characteristics of the areas where the different clades of *Synechococcus* are more abundant, modified from (Scanlan et al., 2009).

Clade	Ecological conditions of largest relative abundance
I	Coastal and/or temperate mesotrophic open ocean waters largely above 30°N and below 30°S
II	Offshore, continent shelf, oligotrophic tropical or subtropical waters between 30°N and 30°S
III	Ultraoligotrophic open-ocean waters
IV	Coastal and/or temperate mesotrophic open ocean waters largely above 30°N and below 30°S
V-VI-VII	Relatively wide distribution but in low abundance in various oceanic waters; have been seen to dominate mesotrophic upwelling regions

Genomic features

The average size of *Synechococcus* genomes is ca. 2.5 Mb, the genome is composed of a single circular chromosome with no plasmids. Marine *Synechococcus*, is characterized by a high G+C content (47.7-69.5%) (Watson et al., 1996), much higher than in *Prochlorococcus* (table 4).

Table 6. Most representative *Synechococcus* genomes.

Synechococcus	Subcluster or ecotype[a]	Clade no[b].	Genome size (Mb)	GC (%)
CC9311	5.1B	I	2.61	52
CC9605	5.1A	II	2.51	59
WH8102	5.1A	III	2.43	59
CC9902	5.1A	IV	2.23	54
BL107	5.1A	IV	2.28	54
WH7803	5.1B	V	2.37	60
WH7805	5.1B	VI	2.62	57
RS9917	5.1B	VIII	2.58	65
RS9916	5.1B	IX	2.66	60

[a]5.1A and 5.1B, subclaster number as defined (Dufresne et al., 2008). [b]Ecotype as defined in reference (Fuller et al., 2003; Kettler et al., 2007).

Photosynthetic apparatus

In *Synechococcus* the main light-harvesting antenna is the phycobilisome, this supramolecular complex comprises the phycobiliproteins (for instance phycoerythin and phycocyanin) each of which binds one or several light-harvesting chromophores, such as phycocyanobilin and phycoerythrobilin (Six et al., 2004; Six et al., 2005; Six et al., 2007b). The antenna complex works collecting light and transfers the energy to the photosystem II (PSII) core antenna proteins (CP43 and CP47) and then into the PSII reaction center (comprising multiple proteins and cytochrome b_{559}) (figure 10).

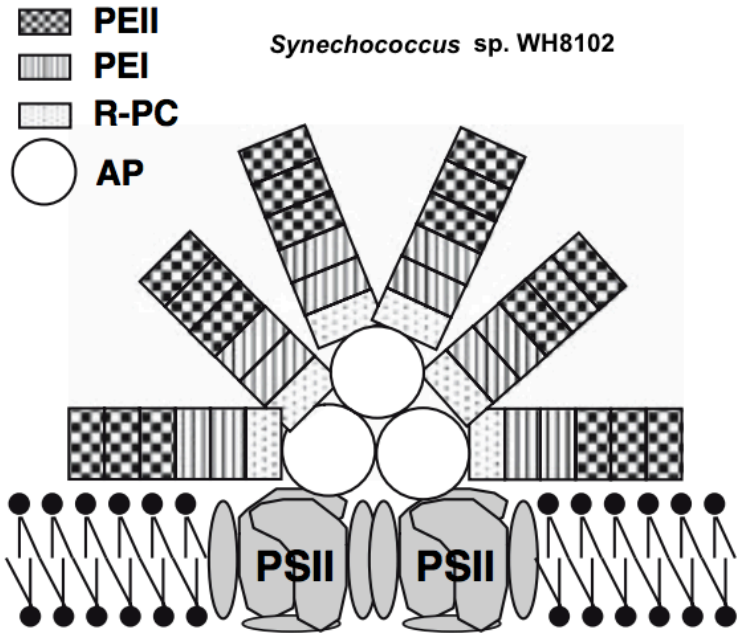


Figure 10. Photosynthetic apparatus of marine *Synechococcus* sp. WH8102. Image modified from (Six et al., 2007b). *PEII: phycoerythrin II. PEI: phycoerythrin I. R-PC: R-phycocyanin. AP: allophycocyanin.

Synechococcus may tune its phycobilisome antenna systems to acclimate to changing temperatures, which may contribute to its greater geographical distribution in the ocean (Six et al., 2005; Six et al., 2007c; Mackey et al., 2013; Pittera et al., 2014; Pittera et al., 2015; Pittera et al., 2017; Partensky et al., 2018).

Synechococcus has developed a variety of pigmentations (Waterbury et al., 1986; Six et al., 2007c; Garczarek et al., 2008; Shukla et al., 2012; Pittera et al., 2014; Sanfilippo et al., 2016; Pittera et al., 2017; Pittera et al., 2018). The interior of the phycobilisome is composed of allophycocyanin, which is connected to the photosystems, and is considered to be surrounded by six to eight rods comprising phycoerythrin and/or phycocyanin. In *Synechococcus*, there are different kind of pigments, called 1, 2 and 3 respectively, the classification criterion is based on the absence (pigment type 1) or presence of one (pigment 2) or two (pigment 3) phycoerythrins (1 and 2). Moreover, pigment type 3 has been subdivided into four subtypes (3a to 3d), depending on the proportion of PUB (phycourobilin) to PEB (phycoerythrobilin, a chromophore found mostly in phycoerythrin but potentially also in phycocyanin (Palenik, 2001)) chromophores linked to phycoerythrins (figure 11) (Six et al., 2004; Six et al., 2005; Six et al., 2007a; Six et al., 2007c).

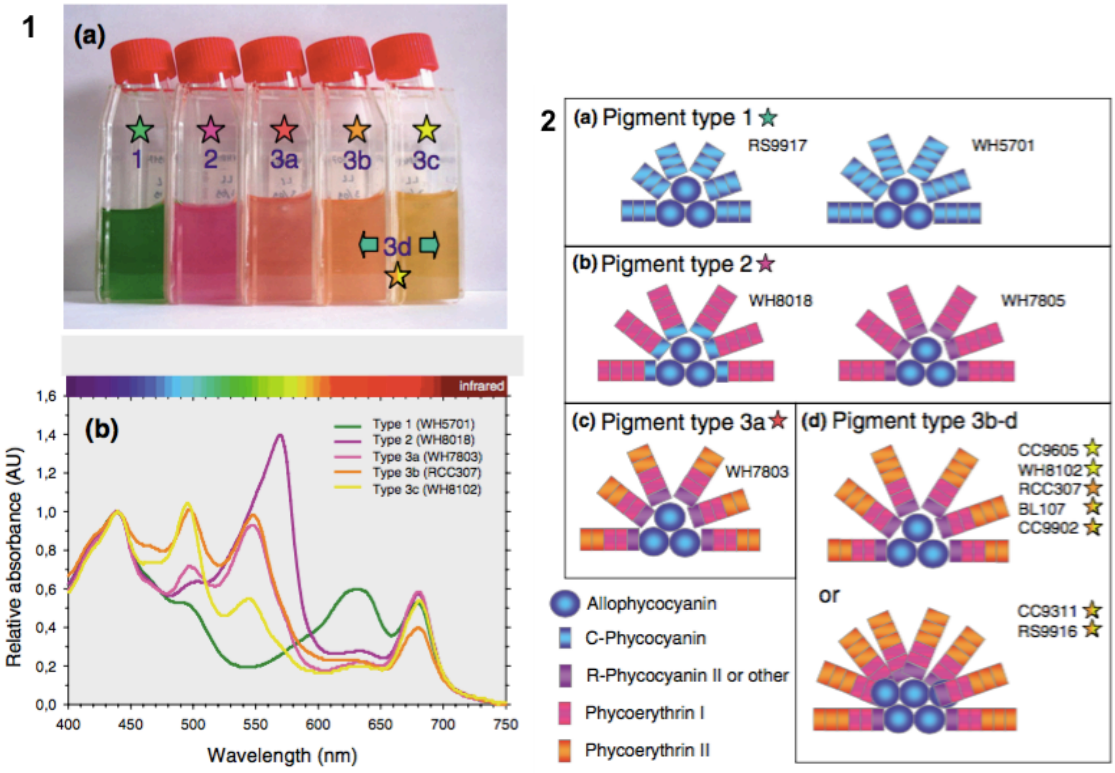


Figure 11. Diversity of pigment in marine *Synechococcus*. 1. (a) Picture of cultures representative of the three main pigment types (1 to 3) and subtypes (3a to 3c). 3d is the pigment type that modify their pigmentation from 3b to 3c. (b) Absorption properties of each pigment. 2. Models of phycobilisome structure from the different pigments types (a-d). Imagen taken from (Six et al., 2007c).

3. CARBON METABOLISM

3.1 Carbon cycle

Carbon and nitrogen are the two most abundant elements for all living organisms, and their metabolism is tightly coupled (Zhang et al., 2018).

The oceans, the atmosphere and the Earth's crust act as carbon reservoirs, with the ocean having the highest amount. In figure 12 a global scheme of the carbon cycle is shown, the values in parentheses are estimates of the main carbon reservoirs in gigatons (GT). The natural flow between the terrestrial biosphere and the atmosphere is on average 120 GT of carbon per year, and that between the oceans and atmosphere is about 90 GT per year (IPCC, 2008).

The biological pump (which includes processes of photosynthesis, feeding, respiration and decomposition) refers to the fraction of organic carbon that is formed in accumulations resistant to degradation and is sunk at the bottom of the ocean (Falkowski and Scholes, 2000).

Biological pumps and solubility are responsible for controlling the amount of carbon that is transported to the depths of the ocean and the exchange of CO_2 produced between the atmosphere and the ocean. Human activities emit around 9 GT of carbon each year, of which 2 GT are removed by the ocean, 3 GT are taken up by natural terrestrial processes and another 4 GT remain in the atmosphere (DOE, 2008).

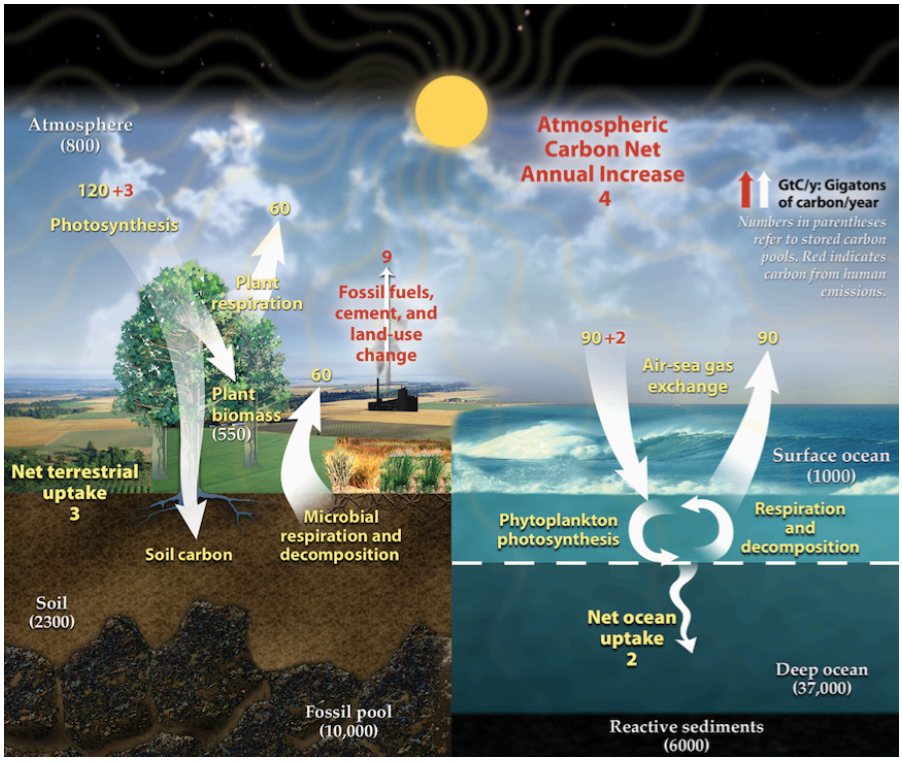


Figure 12. Global carbon cycle.
Picture taken from https://genomicscience.energy.gov/index.shtml.

The amount of phytoplankton in the oceans represents only about 2% of total global carbon in plants, but these organisms fix between 30-50 x 10^9 tons of carbon annually, which represents about 40% of the total (Falkowski et al., 1992; Berges and Harrison, 1995; Falkowski, 2012). The CO_2 is absorbed from the atmosphere and is used for the synthesis of organic molecules, through the processes of photosynthesis (made by algae, green plants and cyanobacteria such as *Prochlorococcus* and *Synechococcus*). The unicellular bacteria *Prochlorococcus* and *Synechococcus* are the most abundant in the ocean and the main participants in the global carbon cycle (Berube et al., 2018).

3.2 Coupling between Carbon and Nitrogen metabolic pathways

Figure 13 summarizes the metabolic coupling of nitrogen and carbon assimilation in cyanobacteria. As for other autotrophic photosynthetic organisms, CO_2 is the main primary carbon source for cyanobacteria, although some cyanobacterial strains can also use a sugar or other organic carbon compound as the carbon source (Rippka et al., 1979; Zhang et al., 1989; Burnap, 2015).

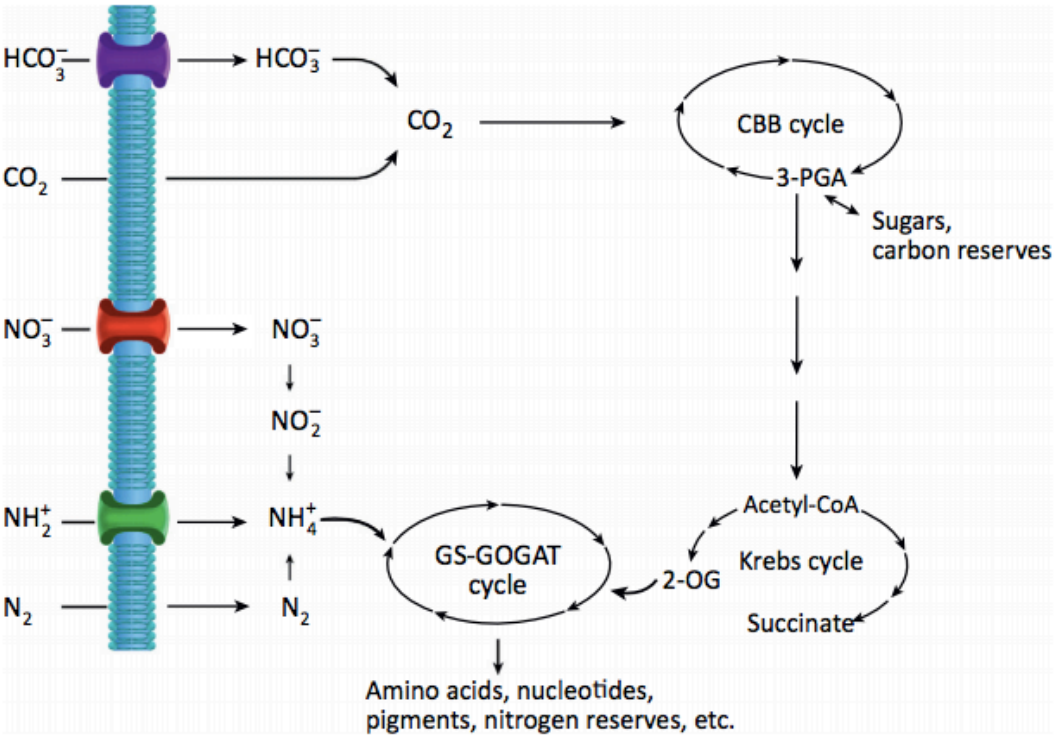


Figure 13. Coupling between Carbon and Nitrogen Metabolism. The inorganic carbon, as CO_2 or HCO_3^- enters the cells and as CO_2 is assimilated into organic carbons through the Calvin-Benson-Bassham (CBB) cycle. Fixed carbon through the CBB cycle is also used to feed the Krebs cycle, which produces intermediates for a variety of carbon compounds. Cyanobacteria can use different forms of inorganic nitrogen, but all of them are assimilated in the form of NH_4^+ through the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle using 2-OG as carbon skeleton. Glu and Gln, are the amino acids produced through the GS-GOGAT cycle, they are also important amino group donors for the synthesis of other nitrogenous compounds. *3-PGA (3-Phosphoglycerate). Picture taken from (Zhang et al., 2018).

3.3 Carbon metabolism in cyanobacteria

Prochlorococcus and *Synechococcus* are the main representatives of marine photosynthetic organisms. They have a high impact on carbon metabolism, due to their participation in the processes of carbon fixation and biomass production. However, at the beginning of the XX century little was known about the metabolism of carbon in these cyanobacteria (García-Fernández and Diez, 2004).

Many studies carried out during oceanographic cruises have confirmed a great level of marine microbial diversity (Rusch et al., 2007; Yooseph et al., 2007; Williamson et al.,

2008). Comparisons of the genomes of multiple photoautotrophic cyanobacteria have been made to study the genetic diversity found in carbon metabolism (Beck et al., 2012). The comparison process was made with respect to the metabolic functionality based on the alignment of protein sequences and on a construction of orthologous gene families. The analysis of the sequences of cyanobacteria, which included 8 freshwater and 8 marine strains, allowed to obtain an outline of the different metabolic pathways present in these cyanobacteria, showing the great metabolic diversity that exists in this group of microorganisms (figure 14).

In the image, we can verify that all the key enzymes of the Calvin-Benson cycle, responsible for the fixation of CO_2, are found in all the strains of cyanobacteria used in the study, as well as the enzymes involved in the route of the pentose phosphate. The enzymes absent in the majority of *Prochlorococcus* strains are indicated in green color. Fructose-1,6-biphosphatase (EC 3.1.3.11) it is not annotated in a multitude of strains, being absent in all the α-cyanobacteria (based on a classification of the rubisco phylogeny) (Beudeker et al., 1980) which include *Prochlorococcus* strains. Although in one of the reconstructions of the carbon metabolism model in *Synechocystis* sp. strain PCC 6803, the enzyme was found to be not essential for biomass formation (Knoop et al., 2010) since it can be substituted by the bifunctional enzyme fructose-1,6-/sedoheptulose-1,7-biphosphatase (EC 3.1.3.37) present in all the strains of the study. The enzyme phosphofructokinase (EC 2.7.1.11) is not annotated in some strains, it is absent in *Prochlorococcus*, phosphofructokinase is essential in glycolysis, so it might be thought that the use of glycogen as an energy source would be carried out by the pentose phosphate route. Other enzymes of glycolysis, such as fructose-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase and pyruvate kinase are listed in all the cyanobacteria studied in this work (Beck et al., 2012).

The phosphoenolpyruvate carboxylase enzyme is essential in the formation of tricarboxylic acid cycle intermediates. It was initially thought that cyanobacteria had the peculiarity of having an incomplete tricarboxylic acid cycle, lacking 2-oxoglutarate dehydrogenase (Stanier and Cohen-Bazire, 1977). However, it is now known that this group of microorganisms can use alternative routes to close the cycle. For instance, in *Synechocystis* sp. 6803 (Knoop et al., 2010) using three enzymes such as glutamate decarboxylase (EC 4.1.1.15), 4-aminobutyrate transaminase (EC 2.6.1.19) and succinate-

semialdehyde dehydrogenase (EC 1.2.1.16); or in *Synechococcus elongatus* sp. 7942, with the enzymes 2-oxoglutarate decarboxylase (EC 4.1.1.71) and succinate-semialdehyde dehydrogenase (EC 1.2.1.16) converting 2-oxoglutarate to succinate and thus closing the tricarboxylic acid cycle (Zhang and Bryant, 2011).

Cyanobacteria, as photoautotrophic organisms, store carbon compounds during the day to maintain cellular function at night. The most abundant storage compound in cyanobacteria is glycogen, synthesized from glucose-6-phosphate, but there are also other less common such as cyanophycine (Beck et al., 2012).

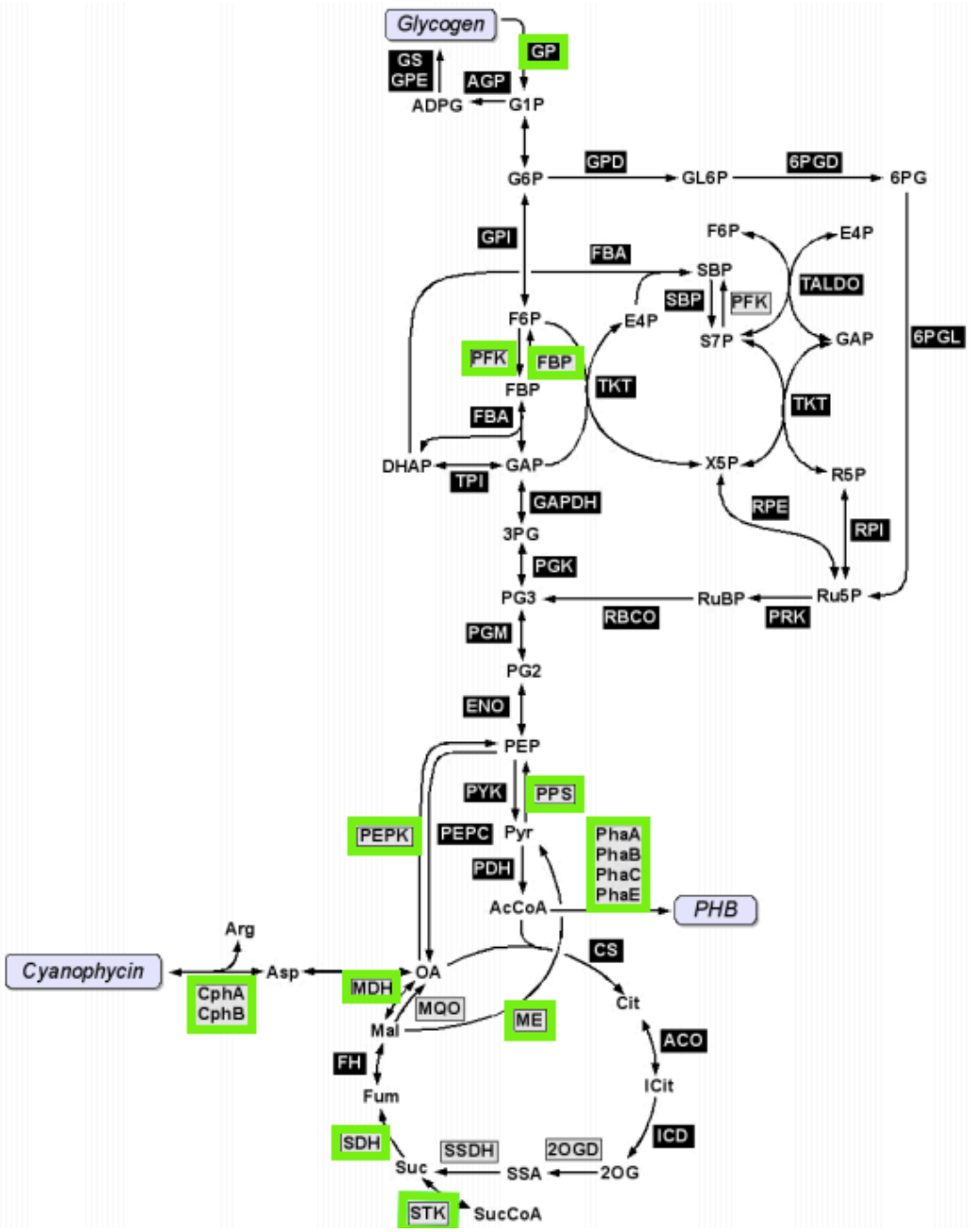


Figure 14. Metabolic pathways of carbon metabolism in cyanobacteria. Black boxes indicate enzymes whose corresponding CLOGs are associated with all 16 cyanobacterial strains studied. The enzymes absent in the majority of *Prochlorococcus* strains are indicated in green color. Picture taken from (Beck et al., 2012).

3.4 Transport of organic compounds

The use of organic compounds has been studied in a large series of cyanobacterial strains (Pelroy et al., 1972; Rippka et al., 1979; Flores and Schmetterer, 1986; Joset et al., 1988; Zubkov et al., 2003; Michelou et al., 2007; Picossi et al., 2013), but not all cyanobacteria have this capability.

The cyanobacterium *Cyanothece* sp. ATCC 5142 is able to use carbon substrates to grow mixotrophically under light conditions and heterotrophically under dark conditions (Reddy et al., 1993). It has been shown that *Cyanothece* sp. ATCC 5142 has a set of genes involved in the metabolism of pyruvate and glucose (Welsh et al., 2008). On the other hand, *Cyanothece* sp. ATCC 5142 was able to use glycerol as carbon source (Feng et al., 2010). There is another cyanobacterium capable of using glycerol to grow heterotrophically, as is the case of *Agmenellum quadruplicatum* PR-6 (Rippka et al., 1979).

It has been described that cyanate can be used as a source of nitrogen in marine cyanobacteria, being an essential nitrogen source in oligotrophic zones for photosynthetic organisms and much less used by non-photosynthetic organisms (Kamennaya et al., 2008b). The cyanate is an ion produced during the decomposition of urea, an organic nitrogen molecule widely used by cyanobacteria; genes coding for a cyanate transporter have been detected in *Prochlorococcus* and *Synechococcus* (Palenik et al., 2003; Kamennaya and Post, 2011). There are other nitrogen compounds of organic origin found in oceanic areas, amino sugars such as chitin and peptidoglycans are recognized as molecules with a high energy power and as an important source of nitrogen for marine microorganisms (Cottrell and Kirchman, 2000; Riemann and Azam, 2002; Yelton et al., 2016). It is considered that the bioavailability of nitrogen limits the productivity of oligotrophic oceans. In order to evaluate the microbial requirement of small molecules of organic nitrogen in the ocean, the rates of microbial absorption of amino acids (leucine, methionine and tyrosine) and amino sugars (glucosamine and N-acetyl-glucosamine) were compared, as well as those of glucose (Zubkov et al., 2008). The results showed a clear preference in oceanic bacterioplankton for amino acids, particularly leucine, compared to amino sugars. (Zubkov et al., 2003; Zubkov et al., 2004; Zubkov and Tarran, 2005; Mary et al., 2008b; Mary et al., 2008a; Zubkov et al., 2008)

An important source of carbon and sulfur for most heterotrophic bacteria is DMSP (dimethylsulfoniumpropionate) (Kiene et al., 2000). This organic nitrogenous compound is used by various cyanobacteria such as *Synechococcus* (Malmstrom et al., 2005). Cyanobacteria can benefit from the DMSP degradation (Kiene et al., 2000), and it can be used by cyanobacteria such as *Synechococcus* and diatoms (Vila-Costa et al., 2006; Ruiz-González et al., 2012). *Prochlorococcus* is capable of transporting and assimilating DMSP, that is an organic compound rich in carbon and sulfur (Vila-Costa et al., 2006), in addition, the light acts as to enhance the transport of DMSP (Ruiz-González et al., 2012).

It has been shown that amino acid transport is enhanced by light, suggesting it is carried out by using an active transport mechanism. This has been demonstrated in some cyanobacteria, as *Synechocystis* sp. PCC 6803 and *Anacystis nidulans*, where it has been shown that the energy used for this active transport comes from the photosystem I (Lee-Kaden and Simonis, 1982; Anderson and McIntosh, 1991).

In addition, recent studies indicate that cells have evolved regulatory mechanisms that minimize transcriptional switching and, unexpectedly, localize essential sulfur acquisition genes in a genome region normally associated with adaption to environmental variation (Smith et al., 2016).

It has been described that the organic compound most used by heterotrophic bacteria is glucose (Moat A.G. et al., 2003). The use of glucose by cyanobacteria was also reported in early cyanobacterial studies, which described glucose uptake in *Synechocystis* PCC 6803, *Synechocystis* PCC 6714, *Nostoc* MAC (PCC 8009) and *Anabaena* sp. PCC 7120 (Pelroy et al., 1972; Rippka, 1972; Beauclerk and Smith, 1978; Raboy and Padan, 1978; Rippka et al., 1979; Flores and Schmetterer, 1986; Joset et al., 1988; Kurian et al., 2006; Ma and Mi, 2008; Nieves-Morión and Flores, 2018). *Synechocystis* sp. strain PCC 6803 has a glucose transporter with a kinetic constant Ks of 0.4 mM and is a model of freshwater cyanobacteria with photoheterotrophic growth in the presence of glucose. The Ks of *Synechocystis* PCC 6803 is very similar to the *Synechocystis* PCC 6714 strain (0.58 mM) which suggests a great analogy between both species (Joset et al., 1988).

The *glcP* gene was reported to encode a fructose-glucose permease in *Synechocystis* sp. PCC 6803 (Zhang et al., 1989; Schmetterer, 1990), surprisingly, a GlcP permease was demonstrated to be necessary for *Nostoc punctiforme* to carry out symbiosis with the plant *Anthoceros punctatus* (Ekman et al., 2013). Moreover, multiple ABC sugar transporters have been shown in Anabaena sp. PCC 7120 (Nieves-Morión and Flores, 2018) suggesting diverse roles for GlcP transporters in free-living and symbiotic cyanobacteria (Picossi et al., 2013).

The livelihood of cyanobacteria is dependent upon light, a comprehensive understanding of metabolism in these cyanobacteria requires taking into account the effects of day-night transitions and circadian regulation (Espinosa et al., 2018; Welkie et al., 2019). The glucose transporter of the freshwater cyanobacteria *Aphanocapsa* sp. PCC 6714 showed a higher glucose transport in the presence of light (Beauclerk and Smith, 1978). It has been shown that in many cyanobacteria the transport of these organic molecules is enhanced by light (Michelou et al., 2007; Gómez-Baena et al., 2008), suggesting that this process is related to the circadian rhythm (Chen et al., 1991). Moreover, Kai proteins globally regulate circadian gene expression of cyanobacteria (Nakajima et al., 2005).

Transport of organic compounds in *Prochlorococcus*

Prochlorococcus was considered as an autotrophic organism for a long time, however different studies indicate that *Prochlorococcus* can be a photoheterotrophic organism (Zubkov and Tarran, 2005; Eiler, 2006; Vila-Costa et al., 2006; Michelou et al., 2007; Gómez-Baena et al., 2008; Mary et al., 2008b; Muñoz-Marín et al., 2013; Muñoz-Marín et al., 2017; Berube et al., 2018; Duhamel et al., 2018).

In the ocean depths, light is scarce and *Prochlorococcus* is very abundant, it is possible that reduced molecules such as amino acids, can be an important source of nitrogen for *Prochlorococcus*, due to its lower metabolic cost (Hedges et al., 2001; Zubkov et al., 2004). Regarding the use of amino acids by *Prochlorococcus*, it has been shown that it is able to use nanomolar concentrations of methionine and leucine (Zubkov et al., 2003; Zubkov and Tarran, 2005; Björkman et al., 2015).

There are certain strains of *Prochlorococcus* (MED4, NATL1A, NATL2A) which have in their genome the gene that codes for cyanase (*cynS*), which carries out the conversion of cyanate to ammonium. Besides, some strains as MED4 (genetically equal to PCC 9511) and SP strain have the genes that code for an ABC type cyanate transporter (Kamennaya et al., 2008a; Maeda and Omata, 2009) and are able to use cyanate as a nitrogen source (Kamennaya et al., 2008b; Kamennaya and Post, 2011). The use of cyanate could provide a competitive advantage to *Prochlorococcus*, allowing it to occupy niches with nitrogen deficiency in oceanic surface areas.

Genomic analysis of *Prochlorococcus* indicated the presence of sugar transporters (Dufresne et al., 2003; Rocap et al., 2003) as the *Pro1404* gene, originally described as a Na^+-galactose transporter (*melB*) (Mizushima et al., 1992). The expression of this gene increased upon glucose addition (Gómez-Baena et al., 2008). The Pro1404/*glcH* encodes a very high affinity glucose transporter in *Prochlorococcus* sp. strain SS120. Moreover, glucose uptake was demonstrated by natural *Prochlorococcus* populations (Muñoz-Marín et al., 2013). Glucose uptake kinetics showed a significant degree of diversity in several model *Prochlorococcus* strains (Muñoz-Marín et al., 2017). Marine picocyanobacteria are actively monitoring the availability of glucose in the ocean, with the purpose to upregulate *glcH* expression in order to exploit the presence of sugars in the environment, suggesting that the transcriptional regulation of glucose uptake has been adjusted by evolutive selection.

In addition, the phylogeny of *glcH* is very similar in *Prochlorococcus* and *Synechococcus*, suggesting that this gene has undergone an evolutionary selection in the different clades of *Prochlorococcus* and *Synechococcus*, which agrees with metagenomic studies that have demonstrated a great capacity of mixotrophy in marine picocyanobacteria (Yelton et al., 2016).

Darkness induced a decrease in glucose uptake of *Prochlorococcus* sp. strain SS120 (Gómez-Baena et al., 2008). Later studies conducted in the same strain showed that addition of photosynthetic electron transport inhibitors, such as DCMU and DBMIB, also inhibited glucose uptake (Muñoz-Marín et al., 2017).

4. METABOLIC BALANCE OF THE OLIGOTROPHIC OCEAN

4.1 Metabolic strategies

Metabolic modes can be defined based on several criteria; depending on the energy source used, on the compound used as an electron donor, and finally, on the carbon source used (table 7).

Table 7. Metabolic strategies (based on (Eiler, 2006))

Source of energy	Electron donor	Carbon source	Denomination
Light	Organic	Organic	Photoorganoheterotroph
		Carbon dioxide	Photoorganoautotroph
	Inorganic	Organic	Photolithoheterotroph
		Carbon dioxide	Photolitoautotroph
Chemical compound	Organic	Organic	Chemoorganoheterotrophic
		Carbon dioxide	Chemoorganoautotrophic
	Inorganic	Organic	Chemolithoheterotroph
		Carbon dioxide	Chemolithoautotroph

The process by which the organic components are used as a carbon source for biosynthesis, is called heterotrophy. On the other hand, autotrophy is a necessary process to carry out the conversion of inorganic matter to organic matter. Phototrophy is defined as the series of processes by which electromagnetic energy is converted into chemical energy (Eiler, 2006). Mixotrophy is the ability to combine autotrophic and heterotrophic modes of nutrition. It is widely spread in various microorganisms, particularly in plankton and cyanobacteria (Matantseva and Skarlato, 2013). Mixotrophy has an important impact on the comprehension of the matter and energy flows in marine ecosystems (Matantseva and Skarlato, 2013).

Mixotrophic bacteria have advantages in aquatic ecosystems, due to their ability to use more resources than other organoheterotrophic or photoautotrophic bacteria.

Mixotrophy provides the bacteria with metabolic benefits, such as increased amount of energy and reducer power. On the other hand, as a result of mixotrophy, the bacterium has to invest resources for uptake and photosynthesis, such as photosynthetic apparatus and proteins for transport and subsequent degradation of organic compounds. In addition, it is considered that a mixotrophic organism could not compete with a phototrophic specialist for light, or a heterotrophic specialist for dissolved organic matter (Eiler, 2006).

4.2 Current controversy about net production in the oligotrophic areas

Oligotrophic areas in the ocean occupy a very large volume (estimated 60% of the surface of the ocean), although its productivity is very low, the contribution to total carbon photosynthetically incorporated in the ocean is approximately 30% (Longhurst, 1995; Marañón et al., 2003; Marañon, 2005; Zwirglmaier et al., 2007). The distribution and concentration of organic carbon is due to the coexistence of several processes that establish the sources and sinks of organic carbon in the ocean. Primary production and respiration represent the largest organic matter sink in the biosphere (Williams and del Giorgio, 2005). Net production depends largely on the amount of carbon that can settle on the ocean floor and the exchanges that take place with the atmosphere (Basu and Mackey, 2018).

For more than a decade there has been controversy in oceanography regarding the metabolic state of the oligotrophic subtropical gyres of the open ocean (Ducklow and Doney, 2013). A group of scientists take the view that these gyres exhibit a state of net autotrophy: their gross primary production exceeds community respiration when the average term considers a widely adequate area and for a long duration (Williams et al., 2013). However, another group of scientists take the opposite view: these gyres are net heterotrophic, with respiration exceeding the primary production (Duarte et al., 2013). The question remains unsolved because the net state is finely balanced between large opposing fluxes and most of the current measurements have large uncertainties (Ducklow

and Doney, 2013). It is difficult to establish a correct quantification of ocean balance between photosynthesis and respiration (del Giorgio and Duarte, 2002). In addition, the estimates are very variable since the carbon flux measurements are subject to methodological problems with differences in the spatial and temporal scale.

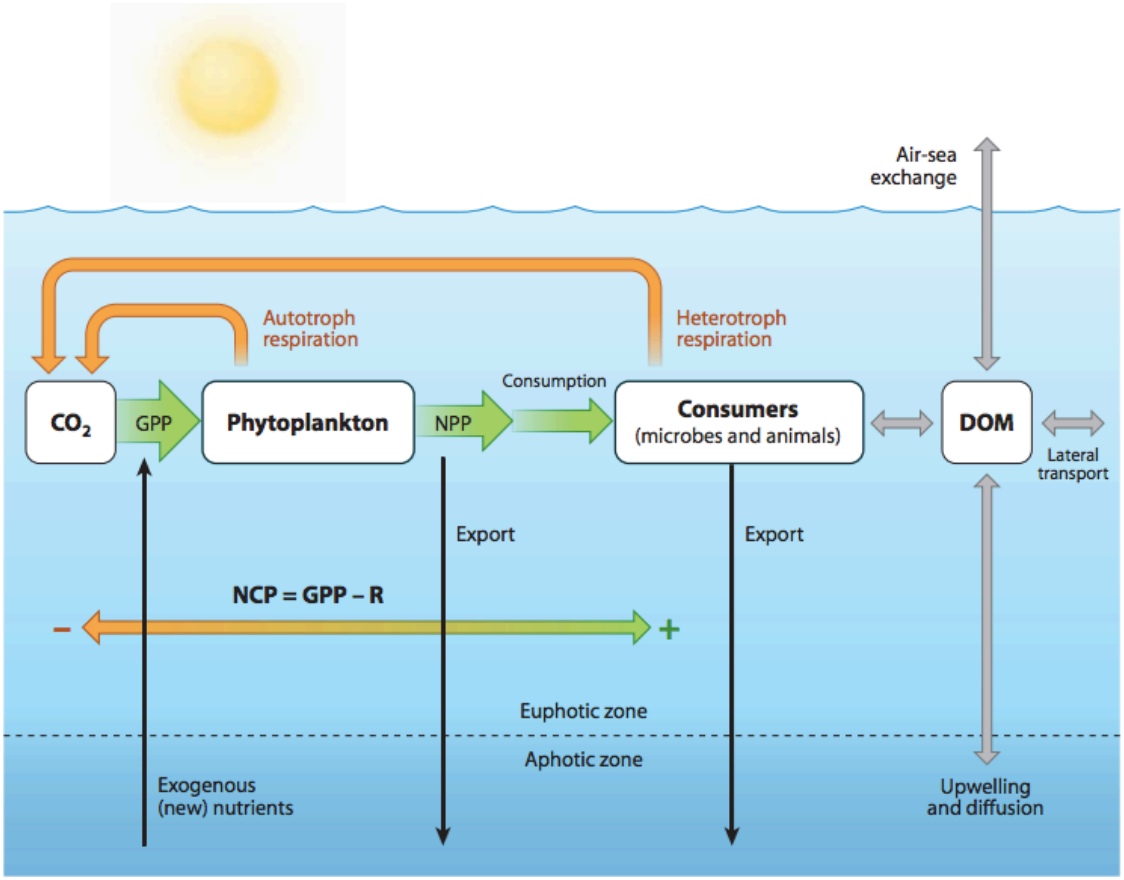


Figure 15. Metabolic exchanges, transformations, and related processes in the surface ocean, all of which influence the balance between net autotrophy and heterotrophy. GPP is the gross primary production, while NPP is the net primary production, NCP is net community production; and R is community respiration. Dissolved organic matter (DOM) includes that produced by phytoplankton and consumers and utilized by heterotrophic bacteria (and some phytoplankton) in situ. Vertical, horizontal, and atmospheric sources of exogenous (new) nutrients (such as nitrogen) support a varying fraction of the GPP, termed new production. This is quantitatively equivalent to the NCP over appropriate time and space scales. Picture taken from (Ducklow and Doney, 2013).

4.3 Proteomic and metabolomic approaches in the marine environment

Proteomics and metabolomics provide systems-level snapshots of the metabolism of a cell or organism at the time of harvest (Viant et al., 2003; Nunn and Timperman, 2007; Sardans et al., 2011).

Proteins are responsible for cellular signals, structural integrity, and catalysis of most biochemical reactions including the production and conversion of the vast array of metabolites required for cellular survival (Poulson-Ellestad et al., 2014).

In marine samples, proteins are present as mixtures of numerous sources in a salt matrix. The advances achieved in biological mass spectrometry now allow the analysis and characterization of the protein component of the marine environment. Proteomics mass spectrometry is an excellent technique for marine studies due to it is a technique allowing analysis of protein mixtures that does not require any prior knowledge of the original protein structures in the mixture. There are several applications derived from marine proteomics, such as the analysis of organisms cultured under different nutrient conditions in order to evaluate expression, cellular adaptation, understand carbon preservation and verify genomic findings with proteomic analyzes to determine which genes are translated and to what extent the protein is expressed. Although important findings have been made in marine studies and mass spectrometry, techniques to achieve the full potential of proteomic studies focused on marine environments have not yet been developed and improved. (Nunn and Timperman, 2007).

When proteomics and metabolomics are jointly taken into consideration, we can get a lot of information on the subject we are studying, since both techniques may characterize the perturbation in metabolites and proteins involved in the same metabolic pathway.

Synechocystis sp. strain PCC 6803 can take up and use external glucose as C-source. Therefore, photoautotrophic and photomixotrophic growth conditions can be directly compared with this cyanobacterium. Transcriptomic and proteomic analyses of glucose effects on *Synechocystis* sp. strain PCC 6803 have been published in the last years (Yang

et al., 2002; Herranen et al., 2004; Kahlon et al., 2006; Ge et al., 2018). Metabolomic fingerprints of glucose addition on the primary metabolism in *Synechocystis* sp. strain PCC 6803 were published (Takahashi et al., 2008; Narainsamy et al., 2013).

In agreement with different studies, proteomics and metabolomics reports revealed that addition of 5 mM glucose under continuous illumination increased the steady state levels of many intermediates of the OPP cycle and glycolysis, whereas the intermediates of the Calvin-Benson cycle decreased. Moreover, the storage of carbon in sucrose and glycogen was stimulated by glucose, while the TCA cycle metabolites did not seem to be much affected (Takahashi et al., 2008; Narainsamy et al., 2013). Remarkably, metabolomic analysis also revealed that glucose addition to *Synechocystis* sp. strain PCC 6803 cells in light induced oxidative stress. Metabolites which are characteristic for cells exposed to oxidative stress were identified in the metabolome of cells grown under photoheterotrophic conditions (Narainsamy et al., 2013).

OBJECTIVES

1. **Study of *glcH* expression in marine picocyanobacteria subjected to different glucose concentrations and light conditions**

2. **Directed mutagenesis of selected amino acid residues of the GlcH protein**

3. **Overexpression of the GlcH protein**

4. **Study of the effect of glucose addition on the metabolome of marine picocyanobacteria**

5. **Study of the effect of glucose addition on the proteome of marine picocyanobacteria**

MATERIALS AND METHODS

Material and Methods

1. STRAINS AND CULTURING

1.1 *Prochlorococcus*

The *Prochlorococcus marinus* strains SS120 (low-irradiance adapted, LL), MIT9313 axenic (low-irradiance-adapted, LL), PCC 9511(high-irradiance-adapted, HL) and TAK9803-2 (high-irradiance-adapted, HL) were used in this study. The strains were provided by the *Roscoff Culture Collection* (*Station Biologique de Roscoff*, France), the *Pasteur Culture Collection* (*Institute Pasteur*, Paris, France) and the *Cyanobacteria Culture Collection* of *Massachusetts Institute of Technology* (Cambridge, United States).

Table 8. Characteristics of the *Prochlorococcus* strains used in this work

Strain	Ecotype	Origin	Depth
PCC9511	HL	Mediterranean Sea	5 m
TAK9803-2	HL	Pacific Ocean	20 m
SS120	LL	Sargasso Sea	120 m
MIT9313	LL	North Atlantic	135 m

Culturing

Prochlorococcus strains were routinely cultured in polycarbonate *Nalgene* flasks (50 mL) using PCR-S11 medium as described (Rippka et al., 2000). The seawater used as the basis for this medium was kindly provided by the Instituto Español de Oceanografía (Spain). The medium was prepared as follows: the seawater was filtered using a bracket with a stainless steel 90 mm diameter filter from *Millipore,* pre-filter of fiberglass (*Millipore*) and a 0.22 μm filter (*Millipore*). The medium was sterilized at 121 ºC and 1 atm of pressure for 20 min. When the sterilized seawater reached the room temperature, the enrichment nutrients solution (table 9) was added. After that, the medium was filtered

again under a sterile laminar flow hood with the filter previously sterilized. The PCR-S11 medium was stored at room temperature.

Table 9. Composition of the PCR-S11 medium. (Final volume 1 L)

Enrichment solution	Volume	Final concentration
Hepes-NaOH 1M pH 7.5	1mL	1 mM
EDTA-Na_2/$FeCl_3$ 2 mM *	1mL	2 µM
$(NH_4)_2SO_4$ 400 mM	1mL	400 µM
Phosphate buffer 50 mM pH 7.5	1mL	50 µM
Trace metals (Gaffron + Se) **	0.1mL	-

* EDTA-Na_2/$FeCl_3$ 2 mM was prepared as follows: 0.54 g of $FeCl_3.6\ H_2O$ in 20 mL of HCl 0.1 N and 0.744 g of Na_2-EDTA in 20 mL of NaOH 0.1 N. Mix them and complete the volume until 1 L. ** See next table.

Table 10. Composition of Gaffron + Se Solution

Trace metals	Stock concentration	Final concentration (nM)
$MnSO_4 \cdot H_2O$	0.6 mM	30
SeO_2	30 µM	1.5
$KAI(SO_4)_2 \cdot 12\ H_2O$	60 µM	3
$VSO_5 \cdot 5\ H_2O$	6 µM	0.3
H_3BO_3	3 mM	150
$Na_2WO_4 \cdot 2\ H_2O$	6 µM	0.3
$Cr(NO_3)_3 \cdot 9\ H_2O$	6 µM	0.3
$NiCl_2 \cdot 6\ H_2O$	30 µM	1.5
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	4.2 µM	1.45
$CuSO_4 \cdot 5\ H_2O$	30 µM	1.5
KBr	60 µM	3
KI	30 µM	1.5
$ZnSO_4 \cdot 7\ H_2O$	60 µM	3
$Cd(NO_3)_2 \cdot 4\ H_2O$	30 µM	1.5
$Co(NO_3)_2 \cdot 6\ H_2O$	30 µM	1.5

Cultures were grown in a culture room set at 24 ºC in conditions of blue irradiances: 4 µE/m^2· s for low-light adapted ecotypes and 40 µE/m^2· s for high-light adapted ecotypes using neon *Sylvania* F18W/154-ST *Daylight,* covered with a filter Moonlight blue L183 from Lee Filters. The strains were kept in liquid culture in flasks of 50 mL *TPP* ® and were refreshed every 5 days with a 1/5 dilution using PCR-S11 medium.

Cryopreservation

Cryopreservation was performed by the methodology described according to (Moore et al., 2007), when the culture was at the end of the exponential phase. An approximate volume of 50 ml of culture was centrifuged at 7,700 x *g* for 15 min and 24 ºC in a sterile tube. The supernatant was eliminated and the pellet resuspended with 1 mL of PCR-S11 medium. This resuspension was transferred to a new *Eppendorf* tube of 1.5 mL. A concentration of 7.5% DMSO (dimethyl sulfoxide) was added, mixed and stored in the freezer at -80 ºC. To revitalize the culture, a small amount of frozen cells suspension was taken from -80 ºC, working under the minimum possible illumination, and 25 mL of fresh medium were added in a laminar flow hood. The cells were then transferred to an approximate volume of 25 mL of PCR-S11 and incubated at 24 ºC in the culture room.

1.2 *Synechococcus*

The *Synechococcus marinus* BL107, WH7803 and WH8102 strains were used in this work. The strains were provided by the *Roscoff Culture Collection* (*Station Biologique de Roscoff*, France.

Table 11. Characteristics of the *Synechococcus* strains used in this work.

Strain	Ecotype	Origin	Depth
BL107	5.1A	Mediterranean Sea	Unknown
WH7803	5.1B	Atlantic Ocean	25 m
WH8102	5.1A	Atlantic Ocean	Unknown

Culturing

Cultures of *Synechococcus* were grown in ASW medium (Artificial Seawater Medium) (Moore et al., 2007). Cells of *Synechococcus* were grown in continuous blue light conditions (40 µE/m^2/s) at 24 ºC. These cultures were grown in 50 mL of

polycarbonate flasks. Dilutions of the culture were made weekly with 1/10 parts of fresh media.

Table 12. Preparation of ASW medium

Components	Final concentration
Turk´Island Salt Mix	
NaCl	481 mM
$MgSO_4 \cdot 7\ H_2O$	28 mM
$MgCl_2 \cdot 6\ H_2O$	27 mM
$CaCl_2 \cdot 2\ H_2O$	10 mM
Kcl	9 mM
Macronutrients	
NaH_2PO_4	50 µM
$(NH_4)_2SO4$	400 µM
Buffers	
$NaHCO_3$	6 mM
Hepes	1 mM
Trace metals	
$Na_2EDTA \cdot 2\ H_2O$	0.1170 µM
$FeCl_3 \cdot 6\ H_2O$	0.1180 µM
$ZnSO_4 \cdot 7\ H_2O$	0.0008 µM
$CoCl_2 \cdot 6\ H_2O$	0.0005 µM

Cryopreservation

In order to preserve the S*ynechococcus* strains, 150 ml of culture were centrifuged at room temperature for 10 min at 10,000 x *g* at room temperature. The pellet was resuspended in 4.436 mL of ASW media with a concentration of 7.5% DMSO.

The resuspension was transferred to a cryogen tube of 5 mL. The cryogen tube was introduced in liquid nitrogen and stored to -80 ºC. Frozen cells were thawed in a water bath at 37 ºC and transferred into 25 mL of sterile medium to revitalize the culture.

1.3 *Synechococcus elongatus* PCC 7942

The *Synechococcus elongatus* PCC 7942 strain was provided by the cicCartuja Biological Cultures Service, Instituto de Bioquímica Vegetal y Fotosíntesis (Sevilla, Spain).

Growth conditions

The cultures were incubated in a thermostated chamber at 30 °C, with white LED light at 25 μE m^{-2} s^{-1}. The growth in liquid medium was carried out in flasks with orbital shaking at a speed of 180 r.p.m. The growth on solid medium was carried out in *Petri* dishes placed inside a methacrylate box located inside the chamber at 30 °C and during the first day without light. After the first day, the cultures were illuminated with fluorescent white light.

Table 13. Recombinants strains of *Synechococcus elongatus* PCC 7942

Recombinant strain	Characteristics
Synechococcus elongatus PCC 7942	WT
Synechococcus elongatus PCC 7942 Mutation aa Asp 52	CK3::Pro1404, Km^r , Mutation aa Asp 52
Synechococcus elongatus PCC 7942 Mutation aa Arg 134	CK3::Pro1404, Km^r, Mutation aa Arg 134

Kanamicin was used at final concentration of 7 μg/mL for selective growth of the corresponding resistant strains.

Culturing

The strain of *Synechococcus elongatus* PCC 7942 and its transformants used in this work were grown in BG11 medium (Rippka et al., 1979). This medium was prepared from a concentrate of salts (100x), K_2HPO_4 and a source of nitrogen (table 14).

Table 14. Preparation of medium BG11

Solutions	Final Concentration
Na_2CO_3	0.2 mM
$MgSO_4$	0.3 mM
$CaCl_2$	0.24 mM
$CoCl_2$	0.2 μM

Citric Acid	28.5 µM
Ferric-ammonium citrate (17%)	0.02 mM
Na_2-EDTA	2.4 µM
H_3BO_3	46 µM
$MnCl_2$	9.1µM
Na_2MoO_4	1.6 µM
$ZnSO_4$	0.8 µM
$CuSO_4$	0.3 µM
K_2HPO_4 (*)	0.2 mM
$NaNO_3$ (*)	17.6 mM

*The media was prepared from a 100x concentrate lacking K_2HPO_4 and $NaNO_3$ which were added before sterilization in the autoclave.

For the solid medium, agar (*Bacto™-Agar, Becton Dickinson* and C*ompany*) was added at final concentration 1% w/v. Agar was dissolved in half of the final volume, the nutrients were separately sterilized and mixed before pouring into the *Petri* dishes. For this purpose, the mixture was cooled to a temperature of 60 ºC and poured into *Petri* dishes (50 mL for each *Petri* dish).

Conservation

Cultures of *Synechococcus* (PCC 7942, and mutants of the *glcH* gene) were stored at 30 ºC in a *Petri* dish with BG11 media.

1.4 *Escherichia coli*

Strains

The strains used routinely were *DH5α* and *HB101,* to prepare competent cells for different transformations. The *Rosetta (DE3)* strain was used to overexpress the protein, this strain has six rare codon tRNAs (AGG, AGA, AUA, CUA, CCC and GGA), in this way it acts facilitating the expression of proteins encoded by rare codons in *E. coli.* The strains were obtained by own elaboration.

Table 15. *E. coli* strains used in this study

Strain	Genotype
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ (*lacZYA- argF*) U169, hsdR17(r_K^- m_K^+), λ–
HB101	F^- mcrB mrr hsdS20 (r_B^- m_B^-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 (Sm^R) glnV44 λ^-
***Rosetta* (DE3)**	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* λ(DE3) [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)

Several other *E. coli* strains were used in attempts to overexpress the GlcH protein, but with low efficiency or negative result, such as *BL21(DE3), BL21-GOLD(DE3), BL21(DE3) PLYSS, C41(DE3)* and *C43(DE3)*. These strains were obtained from the laboratory of Dr. Margarida Archer Oliveira (Macromolecular Crystallography Unit, Oeiras, Portugal).

Culture conditions

E. coli was grown in tubes or flasks at 37 ºC, 200 rpm shaking overnight, the culture media used for the growth of strains of *E. coli* was the liquid medium Luria-Bertani (LB) (Sambrook & Russell, 2001a), which was prepared by dissolving LB Broth *(Pronadisa)* in the amount of distilled water indicated by the manufacturer, and then sterilizing in the autoclave for 20 minutes at 120 ºC. For the preparation of solid medium, LB agar *(Pronadisa*) was used in an equivalent way; after sterilization thereof, it was cooled to a temperature of 50-55 °C and poured into Petri dishes. The antibiotics used were filtered with 0.22 μm of *Millipore* filter.

Conservation

The different strains of *E. coli* were conserved at –80 ºC in its corresponding culture medium with 15% glycerol.

2. CELL COLLECTION AND PREPARATION OF CELL EXTRACTS

2.1 *Prochlorococcus* cell collection

When cultures reached A_{674} of 0.05-0.06, they were centrifuged at 22,000 x *g* for 8 min at 4 ºC using the centrifuge *Avanti J-25 Beckman* with a JA-14 rotor. Later, the supernatant was carefully removed with pipette, and the pellet was resuspended with the corresponding buffer volume. For RNA assays, 2.5 L cultures were used distributing 0.5 L of cultures for each condition, and sampling 500 mL at the indicated times; generally 0.5 mL 10 mM of sodium acetate (pH 4.5), 200 mM sucrose and 5 mM EDTA per half a litre of culture. For metabolomics analysis 9 L of culture were used, distributing 3 L of culture for each condition, after 24 h cultures were centrifuged, all the supernatants were completely removed with pipette, and pellets were frozen in liquid nitrogen and stored at -80 ºC. For proteomic analysis 12 L of culture were used, distributing 2 L of culture for each condition; after 24 h cultures were centrifuged, the supernatants were removed with pipette. Afterwards, pellets were resuspended in 2 mL of 25 mM ammonium bicarbonate.

ASSAY	BUFFER COMPOSITION
RNA analysis	10 mM of sodium acetate (pH 4.5), 200 mM sucrose and 5 mM EDTA.
Metabolomic analysis	Not required
Proteomic analysis	25 mM ammonium bicarbonate

- For experiments addressing *glcH* expression, culture bottles were supplemented with different concentrations of glucose (*Sigma)* (1 nM, 5 nM, 100 nM, 1000 nM).

- For metabolomics and proteomic studies, concentrations of glucose 100 nM and 5 mM were used.

- For experiments requiring darkness, culture bottles were covered with several layers of aluminium foil.

To carry out these experiments, the incubation time with glucose was 24 hours, after which the cells were centrifuged and frozen at -80 ºC.

2.2 *Synechococcus* cell collection

When cultures reached 0.1 absorbance at the wavelengths indicated in Table 16, cells were centrifuged at 22,000 x *g* for 8 min at 4 ºC with an *Avanti J-25 Beckman* centrifuge and a rotor JA-14. RNA buffer was used to resuspend the pellets.

The experiments were carried out in the same way as for *Prochlorococcus*, described in the previous section (2.1).

Table 16. *Synechococcus* strains used in the study and absorbance at which they were measured

Strain of *Synechococcus*	Absorbance
WH7803	550 nm
WH8102	550 nm
BL107	495 nm

2.3 French Press

After thawing the cellular extracts, the cell suspensions were broken in a French pressure cell (*SLM / Aminco model FA-079*) at 10,000 *psi*; the obtained extracts were centrifuged for 30 min at 16,900 x *g* and 4 ºC in an *Eppendorf microfuge 5418R*. The supernatant was transferred to a new tube.

3. ANALYTICAL DETERMINATION

3.1 Determination of the fluorescence spectrum of *Prochlorococcus*

The determination of the fluorescence spectrum of the different *Prochlorococcus* strains was carried out by routinely measuring the emission spectra at 680 nm of the culture samples, with excitation from 375 to 650 nm. To do this, *a Perkin Elmer* fluorimeter, model *LS50B*, with a *101-OS 10* mm quartz cuvette (*Hellma)* was used. The

variation in the composition of photosynthetic pigments of each of the strains allowed to differentiate them on the basis of their fluorescence spectra (Partensky et al., 1993).

3.2 Determination of chlorophyll

To carry out the determination of chlorophyll the method described by Mackinney was used (Mackinney, 1941). Chlorophyll measurements were made using aliquots of cell cultures with different volumes according to the cyanobacteria used.

The samples were centrifuged at 16,100 x *g* for 5 min at room temperature. The precipitate obtained was resuspended in 1 mL of 100% methanol, the samples were vortexed for a few seconds and subjected to a new centrifugation at 16,100 x *g* for 5 min at room temperature. Finally, the absorbance at 665 nm of the supernatant obtained in this last centrifugation was measured. In order to calculate the concentration of chlorophyll in the sample, the absorbance was extrapolated in a straight line standard set up for *Prochlorococcus* by members of our group, taking into account, in each case, the dilution factor of the sample.

The formula used was the following:

$$\mu g \text{ clorophyll/mL} = (A_{665} + 2.996 \cdot 10^{-4}) / 0.074069$$

In the case of *Synechococcus* strain PCC 7942, and the transformants mutated in amino acid residues (Asp52, and Arg134) of the Pro1404 transporter, 100 µL of the culture were taken in an *Eppendorf* tube, and centrifuged 1 min at 16,100 x *g*, the supernatant was removed, and 1 mL of methanol was added to the precipitate. Then, it was vortexed 30 seconds and centrifuged again 1 min at 16,100 x *g*. Absorbance was measured at 665 nm. The concentration of chlorophyll was obtained by the following equation:

$$\mu g \text{ clorophyll/mL} = A_{665} \times 13.43 \times 11$$

3.3 Determination of protein concentration

Protein concentration of soluble fractions was determined by the *Bio-Rad Protein assay* according with the Bradford method (Bradford, 1976). The samples were diluted 1:1000 and the *Bio-Rad Protein assay* was diluted 1:5. After that, we mixed 100 µL of the reagent and 100 µL of diluted sample in a 96 well plate. The reaction was measured at 595 nm using a *Multiskan* system from *Thermo Fischer.* A standard curve was made in all determinations in order to avoid the variability of the reagent by preparing a serial dilution of 1 mg/mL of bovine seroalbumin.

3.4 Determination of nucleic acids concentration

The nucleic acid solutions (DNA and RNA) were quantified by measuring their absorbance at 260 nm using the *NanoDrop ND-1000* spectrophotometer (*NanoDrop Technologies*, Inc. Wilmington, USA). To measure nucleic acids concentration, the program "Nucleic Acid" was selected. 2 µl of *milliQ* water was used as blank, after 2 µl of the sample were pipetted on the measuring pedestal. Sample concentration was determined in ng/µL based on absorbance at 260 nm. The ratios 260/280 and 260/230 were used in order to guarantee the purity of the nucleic acids. The optimum value of the ratio 260/280 had to be between 1.8-2; if it is smaller, it indicates the presence of contaminants such as phenol, proteins or other contaminants that can absorb at or near 280 nm. Ratio 260/230 is another measure that shows nucleic acids purity, the range should be between 1.8-2.2, if it is smaller could indicate the presence of copurified contaminants.

4. MANIPULATION AND ANALYSIS OF NUCLEIC ACIDS

4.1 Plasmids and oligonucleotides used in this work

Table 17. Plasmids not built in this work

Plasmid	Description	Reference	Resistence
pSpark TA	Cloning vector	*Canvax*	Ap^R

pSpark II	Cloning vector	*Canvax*	Ap^R
pcMM-3	Overexpression vector of Pro1404 gene	Muñoz-Marín *et al* 2013	Km^R, Ap^R
pOPINEneo-3C-GFP	Overexpression vector	OPPF-UK pOPIN vectors (unpublished)	Ap^R
pOPINEneo-3C-2STREP	Overexpression vector	OPPF-UK pOPIN vectors (unpublished)	Ap^R
pcAM8	Overexpression vector of Pro1404 gene	Antonio López Lozano (unpublished)	Ap^R

The pcMM-3 vector includes the *Pro1404* gene *Prochlorococcus marinus* SS120 under the control of the strong promoter of the kanamycin resistance cassette C.K3 and a downstream *rrnB* transcriptional terminator. All this interrupting the *asnS* gene (encodes the asparaginyl-tRNA synthetase) of *Synechococcus elongatus* PCC 7942, which has been shown to be dispensable for this cyanobacterium by Dr. I. Luque (Muñoz-Marín et al, 2013).

Plasmids pcMM-3 and pcAM8 were provided my members of our team. The plasmids pOPINEneo-3C-GFP and pOPINEneo-3C-2STREP were supplied by Dr. Margarida Archer Oliveira of the Macromolecular Crystallography Unit, Oeiras (Portugal).

Table 18. Plasmids built in this work

Plasmid	Description	Resistence
pcJA2A	pcMM-3 with mutated Arg134 amino acid residue	Km^R, Ap^R
pcJA4	pcMM-3 with mutated Asp52 amino acid residue	Km^R, Ap^R
pcJA6A	pOPINEneo-3C-GFP with insert of *Pro1404* gene from *Prochlorococcus marinus* SS120	Ap^R
pcJA7A	pOPINEneo-3C-2STREP with insert of *Pro1404* from of *Prochlorococcus marinus* SS120	Ap^R

4.2 Minipreps

Plasmid DNA from 3 mL of recombinant *E. coli* cultures was isolated using the *GenElute Plasmid Miniprep* (*Sigma*) kit, following the manufacturer instructions. 20 µL of water were used as eluent.

4.3 Extraction of DNA from gel

The gel extraction process was carried out with the *GenElute Extraction Kit (Sigma)* for the purification of linear DNA fragments and plasmids, following the kit instructions.

4.4 Enzymatic manipulation of DNA

Digestion with restriction enzymes

The process of digestion was carried out in a total volume of 30 µL. 400 ng of DNA were digested with 0.5 µL (10 U) *SpeI* (*New England BioLabs*), 0.5 µL (10 U) *XhoI* (*New England BioLabs*), 0.5 µL (10 U) *BamHI* (*New England BioLabs*) or 0.5 µL (10 U) *PmeI* (*New England BioLabs*). The buffer used for all restriction enzymes was *Cutsmart* ® buffer 10x. The results of digestion were visualized in a 1% TBE agarose gel.

Ligation

T4 DNA ligase (*dominion MBL*) was used for ligation reactions with the molar relation 3:1 insert:vector, 1 µL of T4 DNA ligase (5 U Weiss/ µL), 1 µL of 10x Buffer T4 DNA ligase. The volume was completed with *milliQ* water until 10 µL and the ligation mixture was incubated between 1 and 24 hours depending on the size of the fragment at a temperature of 22 ºC.

When the fragments of DNA were ligated with *pSpark* vectors the ligation was carried out following the kit instructions.

DNA dephosphorylation

The pcMM3 vector digested with a single restriction enzyme was dephosphorylated to decrease its religation frequency. To do this, it was treated with calf intestine alkaline phosphatase *(CIAP)* from *Roche*, preparing the following reaction mixture for a final volume of 15 µL:1.5 µL of 10x enzyme buffer, 2.5 µL of the enzyme and 11 µL of vector, all incubated for 30 minutes at 37 °C and then at 75 °C for 10 minutes.

4.5 Methods for DNA transfer

Preparation of competent cells

After inoculating 50 mL of LB medium with an isolated colony of *E. coli* that had grown on an LB agar plate overnight at 37 °C, the culture was placed in a rotary incubator at 37 °C, with an agitation of 250 rpm, until reaching an optical density value at 600 nm located between 0.4 and 0.5. Then, it was centrifuged at 2,500 x *g* for 15 minutes, at 4º C, and the precipitate was resuspended in 5 mL of TSS, a preservation solution prepared as follows: 1 g of tryptone, 0.5 g of extract of yeast, 0.5 g of NaCl, 10 g of polyethylene glycol 4000, 5 mL of DMSO and 5 mL of 1 M $MgCl_2$, in 100 mL of distilled water, subsequently adjusting its pH to 6.5, and sterilizing by filtration.

The competent cells prepared with this method were used for transformation. The cell suspension was divided in 100 µL aliquots with 15% glycerol, and frozen in liquid nitrogen. These aliquots were conserved at -80 ºC for later use (Sambrook and Russell, 2001).

Transformation of *Escherichia coli*

The protocol followed for the introduction of the plasmids into the competent cells was based in a transformation by thermal shock. The transformation was carried out after inactivating the ligase enzyme at 65 ºC for 10 min. The volume of ligation (10 µL) was added and mixed with 50-100 µL of competent *E. coli* cells. The ligation mixture was incubated on ice for 30 min, then for 1.5 min at 42 ºC and immediately 5 min on ice. 800 µL of LB medium were added by incubation for 60 min at 37 ºC with shaking (200-

220 rpm). The transformation mixture was poured into LB agar plates and incubated at 37 ºC overnight with the corresponding antibiotic.

Table 19. Composition of LB media

Components	Amount for 1000 mL
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Table 20. Composition of LB Agar

Components	Amount for 1000 mL
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar-agar	15 g

In the experiments to optimize Pro1404 overexpression, the following media were used:

Table 21. Composition of LBlac media

Components	Amount for 1000 mL
LB medium	900 mL
Lactose (40 mg/mL)	100 mL

Table 22. Composition of 2xTY

Components	Amount for 1000 mL
Tryptone	16 g
Yeast extract	10 g
NaCl	5 g

Transformation of *Synechococcus elongatus* strain PCC 7942

Cells of *Synechococcus elongatus* PCC 7942 were centrifuged in sterile centrifuge tube (or 50 mL falcon tube) at 4,000 x *g* for 8 min at room temperature, the cells were washed 2 times with BG11 media, and then the precipitate was resuspended in each wash to eliminate possible extracellular nucleases. The precipitate was resuspended in 4 ml of BG11 medium, and the concentration of chlorophyll was determined in the extract. The volume of the extract was adjusted until a final chlorophyll concentration of 20-100 µg which is equivalent to $1\text{-}2 \cdot 10^9$ cells/mL. 100 µL of the extract were transferred to an *Eppendorf* tube of 1.5 mL, and 300 ng of plasmid DNA were added. It was incubated at room temperature for 5 hours. 200 µL of BG11 were added to the transformation mixture to get a larger volume that facilitates the inoculation in *Petri* dish. The transformation mixture was extended on a sterile filter *Immobilon* (85 mm diameter, 0.45 µm pore, *Millipore*) previously placed on a BG11 media *Petri* dish and allowed to dry for 10-15 minutes in the laminar flow hood. It was placed in the culture chamber at 30 ºC for 48 h with a piece of paper on top of the Petri dish to avoid excess lighting. The filter was transferred to a new Petri dish of BG11 media with kanamycin at a concentration of 10 µg / mL and placed back into the culture chamber under normal lighting conditions (20-25 µE). Then the filter was transferred to new *Petri* dish of BG11 with Km to maintain the selective pressure at all times. The isolated colonies growing on the filter were reseeded in new plates of BG11 with Km. Finally, the mutation introduced in the colonies was checked by PCR.

PCR protocol for checking mutant colonies

The obtention of mutant Pro1404 colonies of *S. elongatus* PCC 7942 was checked by PCR, by using the following PCR mixture: 2.5 µL of 10x buffer *(Biotools)*, 0.8 ng/µL of primers, 2 mM of dNTPs (*Biotools*), 0.5 U of polymerase (*Biotools Taq DNA Polymerase*). The volume was completed to a final volume of 25 µL with *milliQ* water. The standard thermal protocol was: 95 ºC for 5 min (denaturation), followed then by 30-35 cycles of: 95 ºC for 30 s (denaturation), T_m for 30 s (annealing) and 72 ºC for 2.5 min / 3.5 min (extension); a final extension step, 72 ºC for 5 min; and finally hold on at 4 -12 ºC.

4.7 DNA sequencing

The sequencing of DNA fragments was carried out by SCAI (Servicio Central de Apoyo a la Investigación) at the University of Córdoba.

The DNA samples were provided dissolved in distilled water: 15-20 ng/100 bp if it was a PCR product; 300-500 ng in case of being a plasmid. The final volume of the sample, including 3.2 pmoles of the corresponding primer, was 7 µL. The sequences were revised by using *Serialcloner 2-6*-1 and *4Peaks 1.8* software.

4.8 Quantification of gene expression by qRT-PCR

Synthesis of cDNA

cDNA synthesis from 1 µg RNA (DNA-free samples) was done using *qScript cDNA synthesis kit (Quanta)*. The reaction was performed in a final volume of 20 µl, using 0.2 mL PCR tubes following the manufacturer instructions. The thermal program was 1 cycle 22 ºC for 5 min, 1 cycle 42 ºC for 30 min and 1 cycle 85 ºC for 5 min. After cDNA synthesis, 1µL of the obtained mixture was used to PCR amplify the housekeeping gene (*rnpB*), in order to verify that the retrotranscription of the RNA was satisfactory.

Optimization of qRT-PCR amplification

In the process of the optimization of qRT-PCR, the products were verified by PCR amplification of DNA fragments in agarose gel electrophoresis. In order to check the efficiency of the amplifications for each pair of primers, they were analyzed by serial dilutions of samples to test qRT-PCR reactions, in order to achieve a linear amplification. Samples used for the optimization were the amplicon cloned in a *pSPARK TA* or *pSPARK* II vector. Previously, the cloned fragment was sequenced in order to ensure that the amplified product corresponds to the gene of interest.

The standard curve was constructed by plotting the log of the starting quantity of template against the Ct media value (each sample was made in triplicate) obtained during amplification of each serial dilution. Amplification efficiency, (E), was calculated from the slope of the standard curve using the next formula:

$$E = (10^{-1/slope} - 1) \cdot 100$$

The amplification efficiency must be between 90-105%. Reaction efficiencies that reached more than 100% could indicate coamplification of nonspecific products, for example dimers primers. In the case of low efficiencies, it may be due to poor design of primers, or that conditions are not optimal.

qRT-PCR reaction

qRT-PCR of *glcH* and *rnpB* fragments was carried out with an *iCycler iQ System* (Bio-Rad Laboratories) using a *SsoFast™ EvaGreen Supermix*-fluorescence dye (*EvaGreen* dye is a fluorescent nucleic acid dye) based procedure with reagents from *Bio-Rad.* The thermal program was as follows: 95 ºC for 30 s (denaturation), followed by 40 cycles of: 95 ºC for 5 s (denaturation), 58-60 ºC (*Prochlorococcus*) or 58-60 ºC (*Synechococcus*) for 10 s (annealing) and 72 ºC for 10 s (extension).

Table 23. qRT-PCR amplification protocol

	cDNA or plasmid DNA		
Cycling step	Temperature	Time	Cycles
Enzyme activation	95 ºC	30 s	1
Denaturation	95 ºC	5 s	40
Annealing/Extension	58-60 ºC	10 s	
Melt curve	65-95 ºC (in 0.5 ºC)	10 s/step	1

After the final cycle, a melting curve analysis was performed over a temperature range of 65–95 ºC by increments of 0.5 ºC each 10 s in order to verify the reaction specificity.

Analysis

The measurements were made by triplicate in 3 biological replicas under identical conditions. The results obtained from the *iCycler iQ System* were analysed using the software *iCycler iQ v3.0* from (*Bio-Rad* Laboratories).

The relative change in gene expression was endogenously normalized to that of the *rnpB* gene (encoding RNase P) (Holtzendorff et al., 2001), calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001), where Ct is the point at which the fluorescence rises appreciably above the fluorescence background.

Ratio = 2 ΔCt target (calibrator – test)/ 2ΔCt ref (calibrator – test)

$= 2^{-[(Ct, target (test) - Ct target (calibrator)] - [(Ct, ref (test) - Ct ref (calibrator)]} = 2^{-\Delta\Delta Ct}$

5. METHODS FOR PROTEIN ANALYSIS

5.1 Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Gels were run using the system *Mini Protean III* (*Bio-Rad Laboratories)* and 1 or 1.5 mm thick gels.

The composition of the gels is shown in the following table:

Table 24. Composition of gels used in this work

Resolving gel (12%)	**Volume**
Distilled H_2O	3.4 mL
Tris-HCl pH 8,8, 1,5 M	2.5 mL
SDS 20% (w/v)	0.05 mL
Polyacrilamide (30%/0,8% w/v)	4 mL
Ammonium persulfate 10% (w/v)	0.05 mL
TEMED	0.005 mL
Total volume	10.005 mL

Stacking gel (4%)	**Volume**
Distilled H_2O	3.075 mL
0,5 M Tris-HCl pH 6,8	1.25 mL
SDS 20% (w/v)	0.025 mL
Polyacrilamide (30%/0,8% w/v)	0.67 mL
Ammonium persulfate 10% (w/v)	0.025 mL
TEMED	0.005 mL
Total volume	5.05 mL

The samples were prepared using loading buffer 4x *Laemmli Sample Buffer* (*Bio-Rad Laboratories),* final concentration 1x.

The electrophoresis was carried out in a buffer composed of 50 mM Tris base, 30 mM glycine and 2% SDS (w/v), at pH 8.3 and 60 V during the first 20 min, then the voltage was changed to 120 V, and the gel was run to the end, using the system *Power Pac 1000* and *Power Pac 300* from (*Bio-Rad Laboratories).*

Staining of gels

Coomassie Blue stain

To carry out the staining of polyacrylamide gels, a solution composed of: 10% (v/v) acetic acid, 40% (v/v) methanol and 0,1 (w/v) of *Coomassie Brilliant Blue R-250 (Sigma)* was used. To destain the gel, a solution containing 40% (v/v) methanol and 10% (v/v) of acetic acid was used.

5.2 Western Blotting

Semidry transfer of proteins to nitrocellulose membrane

Extracts of *E. coli* (with variable protein concentration) were loaded in different lanes. SDS electrophoresis was carried out using SDS PAGE Running buffer 10x (Tris 250 mM, glycine 1.92 M, SDS 1%). A 1/10 dilution of this buffer was made to run the samples.

Once the samples were run, they were transferred to a nitrocellulose membrane (*Sigma)* utilizing the system semidry *Trans-Blot SD System* (*Bio-Rad Laboratories)*. The process was carried out as follow:

1. After SDS-PAGE put the gel in 100 mL of 1x Transfer Buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) and shake while preparing the nitrocellulose membrane.

2. Cut nitrocellulose membrane with the size of the gel and activate the membrane in 100% methanol. Handle the membrane always with gloves and tweezers avoiding touching the membrane.

3. Cut 2 WB filters (Thick) slightly bigger than the gel size (the bottom filter should be bigger than the top one).

4. Soak the filters in 1x Transfer Buffer.

5. Assemble the "sandwich" (filters+membrane+gel) in the following order in the Western Blot cassette:

   a. Bigger Filter

   b. Activated nitrocellulose membrane

   c. SDS-PAGE Gel

   d. Smaller Filter

6. Pass the roller on top of the "sandwich" to fully remove air bubbles.

7. Put the cassette in the Trans-Blot Turbo and proceed with the Turbo protocol.

8. Remove the membrane with the tweezers and wash in 25 mL TBS-T (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween 20) for 10 min (2x).

The process of transfer was carried out with program *StandardSD* (25 V-1.0 A-30 min).

Incubation with *HisProbe™-HRP Conjugate*

After washing, the membrane was blocked with TBS-T with 5% skimmed dry milk for 60 min and washed twice for 10 min with TBS-T buffer. The incubation with antibody *HisProbe™-HRP Conjugate (Thermofischer)* was done for 1 hour with shaking at room temperature; the antibody dilution used was 1:5000.

Detection with chemiluminescence

After the incubation 2 washes of 10 minutes were made with TBS-T buffer. C The chemiluminescence reaction was produced by adding to the membrane the reagents of *SuperSignal West Pico Chemiluminescent (Thermofischer)*. To detect the chemiluminescence signal, a *ChemiDoc* (*Bio-Rad Laboratories)* system was used. The

obtained images were analyzed with the software *Quantity One 1-D Analysis* (*Bio-Rad Laboratories)*.

6. PURIFICATION OF RECOMBINANT MEMBRANE PROTEIN

6.1 Small scale overexpression test

The overexpression of protein Pro1404 from *Prochlorococcus marinus* SS120 was examined after inducing its expression in *E. coli Rosetta* (DE3) with genotype (F- *ompT hsdS*B(rB- mB-) *gal dcm* λ(DE3) [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (CamR)). The initial growth was adjusted to 0.1 OD in a final volume of 10 mL. 3 hours after induction, cells were harvested by centrifugation at 9,000 x *g* for 30 min at 4 ºC, and the pellets were resuspended in lysis buffer (Tris 100 mM pH 7.5, 100 mM NaCl, 20 mM $MgSO_4$, 1mM DTT) and stored at -20 ºC. The samples were mixed with loading buffer 4x *Laemmli Sample Buffer* (*Bio-Rad Laboratories)*.

6.2 Optimization of overexpression test

To carry out the expression of the Pro1404 transporter, different culture media were tested, with or without IPTG addition to the culture, as well as different growth and induction temperatures.

The first results of the protein expression were obtained using the LB medium, an induction with a final concentration of 0.5 mM of IPTG, with an induction time of 4 hours, and a temperature of growth and induction of 37 ºC. The optimal result was obtained using the LBlac medium (table 21). This medium does not need the addition of IPTG, because it contains lactose, which will cause overexpression of the protein of interest. The optimal growth condition for the expression of the GlcH protein was 21 hours of growth, at a temperature of 22 ºC.

6.3 Culture growth

A variable volume between 100-4000 mL of *E. coli* culture was grown in LBlac media 21 hours at 22 ºC, in the presence of ampicillin or carbenicillin, in a final concentration of 100 mg / mL, to overexpress GlcH from *Prochlorococcus marinus* SS120. After 21 hours of growth, cells were centrifuged at 9,000 x *g* for 30 min at 4 ºC,

resuspended in lysis buffer, and stored at -20 ºC. After the cells were thawed, they were broken using the French press. Samples were passed through the press 3 consecutive times, to ensure total breakage. The next step was to centrifuge the broken cells samples at 10,000 x *g*, the supernatant obtained was transferred to an ultracentrifuge tube and centrifuged at 100,000 x *g* during 60 min at 4 ºC.

6.4 Extraction and solubilization of membranes

With the previous centrifugation, we were able to extract the membrane phase, which was homogenized with the lysis buffer without DTT and $MgSO_4$, that is, the buffer had a composition of Tris 100 mM pH 7.5 and NaCl 100 mM.

To carry out the solubilization of the overexpressed protein, a solubility test was carried out with several detergents, of different compositions, in order to determine which was the optimum for this process. The detergents used were:

-Tritón x 100

-n-Dodecyl β-D-maltoside (DDM)

-Decil maltoside (DM)

All detergents were used at a concentration of 1%, applied to the solution with the resuspended membranes. The samples were kept with the detergent in agitation with a temperature of 4 ºC, during a period of 24 hours. Once the time elapsed, the samples were centrifuged for 60 min at 14,000 x *g* and 4 ºC. The supernatants were transferred to a new *Eppendorf* tube, and the pellets were resuspended in 100 µL of a chaotropic agent solution (8 M urea). Afterwards, each of the phases were analyzed by Western blot.

7. NUCLEIDS ACIDS MANIPULATION AND ANALYSIS

7.1 Genomic DNA isolation

To carry out the genomic DNA extraction of cultures of *Synechococcus* and *Prochlorococcus*, 50 ml of culture in exponential phase, were centrifuged at 27,200 x *g* for 8 min. The obtained supernatant was removed, and the pellet was resuspended with

400 µL of buffer TE (table). The resuspension was transferred to an *Eppendorf* tube with 225 µL of chloroform:isoamyl alcohol (24:1), 20 µL of 10% SDS, 225 µL of phenol (pH 6.0) and 150 µL of glass beads *B. Braun Melsungen AG* (0.10 - 0.11 mm of diameter). The following treatment was carried out: 1 min vigorously vortex- 1 min on ice, the treatment cycle was performed for 4 consecutive times. Then, the mixture was centrifuged at 15,700 x *g*, at 4º C for 15 min. The upper phase was transferred to a new *Eppendorf* tube with 250 µL of chloroform: isoamyl alcohol (24:1) and 250 µL of phenol (pH 6.6), the mixture was mixed and centrifuged at 15,700 x *g* for 10 min at 4 ºC. The new upper phase was transferred to other *Eppendorf* tube, and was filled up with cold ethanol, stored at -20 °C for 2 hours, then the samples were centrifuged at 5,700 x *g*, at 4 ºC for 30 min. The supernatant was eliminated and the pellet dried during 2 hours, the pellet was resuspended in 50 µL of *milliQ* sterilized water and kept at 4 ºC. The obtained genomic DNA was quantified with a *NanoDrop* system and visualized in 1% or 1.7% agarose gels.

Table 25. Composition of TE buffer

Composition	Volume (µL)
Tris-HCl 50 mM pH 7.5	400
EDTA 100 mM pH 8	2
milliQ sterilized water	800

7.2 RNA isolation

To carry out the extraction of genomic RNA from cultures of *Prochlorococcus* and *Synechococcus*, the *Trisure* kit *(Bioline)* was used. To start RNA extraction, 250 µL of cell extract were transferred to an *Eppendorf* tube and centrifuged at 16,900 x *g* for 1 min at 4º C. The obtained pellet was resuspended with 100 µL of lysozyme (50 µg/µL) and incubated for 5 min at room temperature. Then 800 µL of *Trisure* (*Bioline*) were added and mixed by vortex during 15 s. Samples were incubated at room temperature during 15 min at 65 ºC, and then centrifuged at 16.900 x *g*, for 5 min at 4ºC. The supernatant was transferred to a new *Eppendorf* with 200 µL of chloroform. The samples were vortexed for 15 s and incubated for 10 min at room temperature and after were centrifuged at 16,900 x *g* for 10 min at 4 ºC. The samples were separated into several phases; phenol-chloroform phase, an interphase, and an upper phase, which lacked color, and contained

RNA; this phase was transferred carefully to a new *Eppendorf* tube and was filled with 100% isopropanol in order to precipitate the RNA. The samples were incubated for 15 min at room temperature and centrifuged at 16,900 x *g* for 20 min at 4 ºC. The supernatant was eliminated. The pellets were washed with 750 µL of cold ethanol, mixed and vortexed vigorously, and centrifuged at 7,500 x *g* for 5 min at 4 ºC. An additional precipitation process with LiCl 8 M was carried out to improve the quality of the RNA. The next day, 750 µL of 75% cold ethanol were added to each sample, and were centrifuged at 7,500 x *g*, 5 min at 4 ºC. Pellets were resuspended with 20 mL of sterilized *milliQ* water, and RNA samples were stored at -80 ºC for later studies.

7.3 Treatment with DNAse

Once the RNA extraction was done, it was necessary to carry out a treatment with DNase I (RNase- free) (*Ambion*) to degrade the DNA, and thus maintain the integrity and quality of the RNA. 2 µL of 10x *DNase I Buffer* and 1 µL of *rDNase* I were added to a sample containing 1000 ng of RNA and mixed. After samples were incubated for 40 min at 37 ºC, 2 µL of *DNase Inactivation Reagent* were resuspended in the mixture, and incubation was carried out for 10 minutes at room temperature. Then samples were centrifuged at 16,900 x *g*, for 5 min at 4 ºC. The RNA was transferred to a new *Eppendorf* tube, and 1µL of the RNA was used to verify by PCR that the RNA was free of any contaminant DNA.

7.4 Determination of concentration and quality of RNA

After finishing the extraction, the RNA was quantified with a *NanoDrop* spectrophotometer (3.4). Samples quality was checked by visualization after electrophoresis in 1% and 1.7% agarose gels.

7.5 Design of oligonucleotides

To carry out the design of primers, the sequences of genes were obtained from Cyanobase (Cyanobacteria gene annotation database http://genome.microbedb.jp/cyanobase/).

The oligonucleotides used in this work were designed with the sofware *Primer3Plus* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and *AmplifX (https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr)*. In some cases, their design was manually refined. Moreover, the requested conditions were as follows: each pair of primers with T_m (melting temperature) as similar as possible, similar percentage of G+C, absence of secondary structures (cross dimers, self dimers) and an approximate length of 20 nucleotides. The oligonucleotides were provided by Sigma.

Oligonucleotides for qRT-PCR

The optimal T_m of each pair of primers was selected doing a temperature gradient PCR. The amplified product did not exceed 250 nucleotides. The T_m for oligonucleotides for qRT-PCR was between 58 ºC and 60 ºC, for both *Prochlorococcus* and *Synechococcus*.

The oligonucleotides used are shown in the following table for each cyanobacterial strain:

Table 26. Oligonucleotides used for each cyanobacterial strain

Prochlorococcus SS120

Oligonucletides	Sequence 5´-3´	Gene
FST4	GCTTTTATGGCAGGTTCTTT	*glcH*
RST4	CAAATAGCCGCAAGACTCAG	*glcH*
SRF	CTCTCGGTTGAGGAAAGTC	*rnpB*
SRR	CCTTGCCTGTGCTCTATG	*rnpB*

Prochlorococcus PCC 9511

Oligonucletides	Sequence 5´-3´	Gene
PMM1323-5-2	GTCTAGCCGCCACACAATTT	*glcH*
PMM1323-6-2	TGCAGCAATTAGCATCCAAG	*glcH*

FE	ACAGAAACATACCGCCTAAT	*rnpB*
RE	ACCTAGCCAACACTTCTCAA	*rnpB*

Prochlorococcus TAK9803-2

Oligonucletides	**Sequence 5´-3´**	**Gene**
GLCH TAK9803-2 F	ACTGCATCCCATATCTTTATTAA	*glcH*
GLCH TAK9803-2 R	ACGCAATTTGGTTTTTTTCT	*glcH*
FE	ACAGAAACATACCGCCTAAT	*rnpB*
RE	ACCTAGCCAACACTTCTCAA	*rnpB*

Prochlorococcus MIT9313

Oligonucletides	**Sequence 5´-3´**	**Gene**
PMT1398-F*	GGGCTTTACCTGTTGCTCTG	*glcH*
PMT1398-R*	CAAGCAGCGATCCATAGACA	*glcH*
rnpB MIT9313-F	AAGACGAGCTTGGTTGAGGA	*rnpB*
rnpB MIT9313-R	CTCTTACCGCACCTTTGCAC	*rnpB*

Synechococcus BL107

Oligonucletides	**Sequence 5´-3´**	**Gene**
GlcH BL107 F	ATCATGCTGGTGGGTCTAGG	*glcH*
GlcH BL107 R	GTGTACAGCCCGGCAGGT	*glcH*
rnpB BL107 F	CAAGGCCAAGGAACGATG	*rnpB*
rnpB BL107 R	GCAGAGGGTGGGTGGTTAT	*rnpB*

Synechococcus WH7803

Oligonucletides	Sequence 5´-3´	Gene
SYNW0583-F	CTATACCGCCTGGATGGTGT	*glcH*
SYNW0583-R	AATCAGACCCATGCAGATCC	*glcH*
RT-FRNSY	TGAGGAGAGTGCCACAGAAA	*rnpB*
RT-RRNSY	GTTTACCGAGCCAGCACCT	*rnpB*

Oligonucleotides used for cloning

The oligonucleotides used for cloning have the same properties that the oligonucleotides for qRT-PCR, however the amplified product had to be a complete gene from *Prochlorococcus.* The lowercase and underlined nucleotides indicate the mutation introduced in their sequence.

Table 27. Oligonucleotides used for cloning

Oligonucleotides	Sequence 5´- 3´	Function
Pro1404-3	TCTTGCGGCTATTTGGTG	Mutation Arg134 Pro1404
Pro1404-6	AGTGCCTTAATGCGGCCATTCT	Mutation Arg134 Pro1404
Pro1404-12	TCGAGTTCcTATTGAAGTGTCTT	Mutation Arg134 Pro1404
Pro1404-13	CTTCAATAgGAACTCGACTTAACGC	Mutation Arg134 Pro1404
Pro1404-14	TAGAGGAcCATTTATTGCATCCCA	Mutation Asp52 Pro1404
Pro1404-15	TGGGATGCAATAAATGgTCCTCTA	Mutation Asp52 Pro1404
7942_asnS-4R	TGACGGGAGGTATTGGAGTTCTC	Checking the construction of the mutants of Pro1404
CK1-2	GGGATCTCATGCTGGAGT	Checking the construction of the mutants of Pro1404
Pro1404-17	gtttaaacATGCTTTCCTATGGATTA	Amplification of Pro1404 (cutting side PmeI)
Pro1404-18	gtttaaacccTAAGTTGTTTATCTGGTA	Amplification of

		Pro1404 (cutting side PmeI)

Generic oligonucleotides

To verify the ligations, and the sequences in the cloning process, the following primers were used:

Table 28. Generic oligonucleotides used to verify sequences

Oligonucleotides	Sequence 5´- 3´
T7	TAATACGACTCACTATAGGG
SP6	TATTTAGGTGACACTTATAG

Polymerase Chain Reaction (PCR)

To perform PCR reactions, 3 different thermocyclers were used: *T100™ Thermal Cycler* (*Bio-Rad Laboratories), C100 Touch™ Thermocycler* (*Bio-Rad Laboratories)* and *iCycler iQ Multicolor Real Time PCR Detection System* (*Bio-Rad Laboratories)*, by using 2 different polymerase enzymes, provided by *Biotools (Taq DNA Polymerase)* and *Bioline (Mytaq DNA Polymerase)*.

When *Biotools Taq DNA Polymerase* was used, the protocol was: 2.5 µL of 10x buffer *(Biotools)*, 0.8 ng/µL of primers, 2 mM of dNTPs (*Biotools*), 0.5 U of polymerase (*Biotools*). The volume was completed to a final volume of 25 µL with *milliQ* water.

When *Bioline MyTaq DNA Polymerase* was used, the protocol was: 5 *µL 5x Hi-Fi Reaction Buffer*, 0.8 ng/µL primers, 0.5 U of polymerase (*Mytaq*) and was completed to a final volume of 25 µL with *milliQ* water.

With both enzymes, either plasmid, genomic DNA, cDNA or centrifuged cells were used as DNA template. In the case of centrifuged cells, the protocol was: 6 mL of cells were centrifuged at 16,900 x *g,* for 5 min at 4 ºC, the supernatant was removed and the pellet resuspended in 30 µL of water, then the cells were boiled at 100 ºC for 10 min, centrifuged at 16,900 x *g,* for 3 min at 4 ºC, and the supernatant was used as substrate for the PCR.

For *Biootools Taq DNA Polymerase* the standard thermal protocol was: 95 ºC for 5 min (denaturation), followed then by 30-35 cycles of: 95 ºC for 30 s (denaturation), T_m for 30 s (annealing) and 72 ºC for 30 s (extension); a final extension step, 72 ºC for 5 min; and finally hold on at 4 -12 ºC.

For *Bioline Mytaq DNA Polymerase* the standard thermal protocol was: 98 ºC for initial denaturation, followed by 25-35 cycles of: 95 ºC for 30 s (denaturation), T_m for 30 s (annealing) and 72 ºC for 15-30 s (extension); an optional step of extension, 72 ºC for 4-10 min; and finally hold on at 4-12 ºC.

A high fidelity DNA polymerase, *Velocity* (*Bioline*) was used for cloning, since it has corrective activity and the possibility of generating mutations is lower. The buffer used was 5x *Hi-Fi* Buffer (contains 10 mM Mg^{2+}), 0.4 µM final concentration for each primer, 2 mM of dNTPs and 0.5-1U *Velocity DNA Polymerase* was performed in a final volume of 25 µL or 50 µL. The program used was 98 ºC for initial denaturation during 2 min, followed by 25-35 cycles of: 98 ºC for 30 s (denaturation), T_m for 30 s (annealing) and 72 ºC for 15-30 s (extension); and an optional step of extension, 72 ºC for 4-10 min; and finally hold on at 4 ºC-12 ºC.

Electrophoresis of nucleic acids in agarose gel

Samples were subjected to electrophoresis on agarose gels with a concentration of 1% or 1.7% of agarose, depending of the size of the amplified fragment, using *GelRed* (*Biotium)* to visualize it under UV light.

The samples were prepared with 10 % (v/v) loading buffer (0.25% (w/v), bromophenol blue, 0.25% (w/v), Xylene cyanol and 30% (v/v) glycerol). The electrophoresis was carried out in a *BioRad* system (7x10 cm *Mini-Sub Cell GT* and 15x10 cm *Wide Mini-Sub Cell GT*) by using the buffer TBE 0.5x from TBE 2.5x (Table 29) diluted 5 fold, for 60 min at 100 V. The markers used to determine the band size were 100-1000 bp (*gTPbio*) and 250-10,000 bp (*gTPbio*).

Table 29. Composition of TBE 2.5x buffer

Components	Amount for 1 L
Tris base	27 g
Boric Acid	13.75 g
EDTA 0.5 M pH 8	10 mL

A *Molecular Imager ChemiDoc XRS + Imaging System (Bio-Rad Laboratories)* was used to visualize images, with gels subjected to UV illumination (302 nm). The images obtained were analysed with *Quantity One 1-D Analysis Software* (*Bio-Rad Laboratories).*

8. PROTEOMICS

8.1 Protein extracts

Samples were collected as described in section 2.1. After thawing, the cell suspensions were broken as described in section 3.1. Protein concentration was measured by using the Bradford method in a 96 well plate using extracts dilutions (section 3.3). The quality of extracts was checked by SDS-PAGE (section 5.1).

8.2. Trypsin digestion

In solution

Samples containing 100 µg of protein were incubated with *Rapigest* (*Waters Corporation*) at a final concentration of 0.05 % (w/v) for 10 min at 80 °C. Protein samples were then reduced with 3 mM dithiothreitol (DTT) for 10 min at 60 °C, followed by alkylation with 9 mM iodoacetamide for 30 min in the dark at room temperature. Finally, trypsin was added and incubated overnight at 37 °C. To stop the proteolytic reaction and to inactivate and precipitate, the detergent TFA (trifluoroacetic acid) (final concentration 0.5 % (v/v)) was added, followed by incubation for 45 min at 37 °C. To remove all insoluble material, samples were centrifuged at 13,000 *g* for 15 min at room temperature.

In gel

A small (1 mm^3) plug of a stained protein band was cut. The plug was washed alternately with 25 mM ammonium bicarbonate and 25 mM ammonium bicarbonate/acetonitrile (2:1). The gel plug was incubated for 15 min at 37 º C until it was destained. Sample was reduced by adding 25 µL of a 1.5 mg/mL solution of DTT (ca. 10 mM). Then it was incubated at 60 ºC, 60 min, and cooled to room temperature and quick spin to return liquid to the bottom of the tube was made. The liquid around the gel plug was aspirated using a pipette and discarded. The reduction step was followed by alkylation adding 25 µL of a 10 mg/mL solution of iodoacetamide (60 mM). The mixture was incubated at room temperature in the dark for 45 min. The plug was washed with 25 mM ammonium bicarbonate and then it was washed with acetonitrile to dehydrate the plug. At each cycle, the gel plug was incubated for 15 min at 37 ºC. Finally, 10 µL of trypsin (12.5 ng/µL in 25 mM ammonium bicarbonate) was added to the gel plug in an *Eppendorf* tube for digestion. The mix was incubated for 12-16 h (overnight) at 37 ºC. The last step was the peptide recovery by centrifugation. Formic acid (10% v/v) was added to attain a final concentration of 1% (v/v).

8.3 Mass Spectrometric Analysis

LC MS/MS: *Fusion*

All the samples were analyzed as tryptic peptides, resolved by high-resolution liquid chromatography (*U3000 Thermo Fisher*) prior to tandem mass spectrometry. The *Fusion* system (*Thermo Fisher*) was operated in data-dependent acquisition mode. Samples containing between 500 ng and 1µg of protein were analysed and the 10 most intense multiply charged ions were isolated and sequentially fragmented. Precursors selected were dynamically excluded for 20 s. As peak list generating software *Proteome discoverer 2.1* (*Thermo Fisher Scientific*) was used using default parameters. The peak lists obtained were searched against a database composed of reviewed entries of *Prochlorococcus* and *Synechococcus* Uniprot database (January, 2019), using Sequest HT as search engine. Default search parameters were set as follows: methionine oxidation as variable modification, carbamidomethylation of cystein as fixed modification, one trypsin missed cleavage, a mass tolerance of 10 ppm for precursors and 0.01 Da for

fragment ions were allowed. The false discovery rate (FDR) was calculated using the decoy database in Sequest HT.

The peptide mixture (500 ng-1 μg) was trapped onto an *Acclaim PepMap RSCL,* precolumn (300 μm id, 5 mm long, 5 μm particles) *Thermo Scientific*) over 3 min, at a flow rate of 5 μL/min in 2% (v/v) acetonitrile /0.05% (v/v) TFA (trifluoroacetic acid). Bound peptides were resolved on a C18 Nanocolumn *Acclaim PepMap RSCL* (75 μm id, 500 mm long, 2 μm particles) (*Thermo Scientific*) at 300 nL/min over a 60 min linear gradient from 4 to 35% (v/v) acetonitrile in 0.1% v/v formic acid.

All the mass spectrometry analysis were carried out at the Proteomics Service of SCAI (Servicio Central de Apoyo a la Investigación), Universidad de Córdoba.

Proteomic data analysis

LC-MS/MS data were processed for relative label-free quantification using *Progenesis LC-MS* (*Nonlinear Dynamics*) using default parameters.

9. METABOLOMICS

Cyanobacterial cell samples for metabolomic analysis were frozen in liquid nitrogen and stored at -80º C. Once the samples for all strains were available, they were sent to the European headquarters of Metabolon (Potsdam, Germany), which performed the metabolome determinations and the quantitative analysis of data.

Metabolon uses the *DiscoveryHD4* platform, which consists of combining multiple mass spectrometry methods and a LIMS system with a large reference library of metabolite standards and a suite of informatics and quality control software. This allows to automatically and rapidly identify and quantitate metabolites. Metabolon performed the determination of the concentration of the different metabolites, as well as a biostatistical analysis of the results.

10. SOFTWARE

In this work, several databases and software with different purposes were used, shown in table 30.

Table 30. Software and databases used in this work

Software/Webpage	Source/URL	Application
CyanoBase	http://genome.microbedb.jp/cyanobase/	Sequence information
CYORF	http://cyano.genome.jp	Sequence information
Blast	https://blast.ncbi.nlm.nih.gov/Blast.cgi	Sequence information
Primer3Plus	http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi	Oligo design
OligoEvaluator	http://www.oligoevaluator.com/LoginServlet	Quality evaluation of oligos
AmplifX	https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr	Oligo design
Amplify 4	https://engels.genetics.wisc.edu/amplify/	Oligo design
Serial Cloner 2.6.1	http://serialbasics.free.fr/Serial_Cloner.html	Processing of DNA sequence data, primer design and administration of primer and vector database
In silico PCR amplification	http://insilico.ehu.es/PCR/	In silico oligo test for PCR
iCycler IQ v3.0	*Bio-Rad*	Analysis of gene-expression data
ClustalW2 EMBL-EBI	https://www.ebi.ac.uk/Tools/msa/clustalw2/	Multiple sequence alignment
Quantity one	*Bio-Rad*	Image analysis/ Quantification of bands
4Peaks 1.8	https://nucleobytes.com/4peaks/index.html	Analysis of the files generated by the sequencer
Progenesis LCMS	*Nonlinear Dynamics (Waters Corporation)*	Label-free quantification

Proteome discoverer 2.1	*Thermo Scientific*	Peak list generator, protein identification and visualization of protein lists
Sequest	*Thermo Scientific*	Search engine for MS and MS/MS data
Snapgne Viewer	https://www.snapgene.com/snapgene-viewer/	Make plasmid maps

11. STATISTICAL ANALYSIS

Experiments were carried out at least with three independent biological samples. The results are shown with error bars corresponding to the standard deviation. Significance of data was assessed by using the Student's T test, indicated in figures with asterisks: * indicates $p \leq 0.05$; ** indicates $p \leq 0.01$; *** indicates $p = 0.0001$; **** indicates $p < 0.0001$.

12. CHEMICALS

All chemicals were of reagent grade, obtained from *Merck, Sigma, Promega, Novagen, Ambion, Panreac, Thermo Fisher Scientific, Bio-Rad, Amersham Biosciences/GE Healthcare.*

13. APPLIANCES AND INSTRUMENTS

Culture incubation

The growth of marine *Prochlorococcus* and *Synechococcus* cultures was carried out in two large volume culture chambers, which maintained the conditions indicated in sections 1.1 and 1.2. *Synechococcus elongatus* was cultured in an *Algaetron AG 230* growth chamber (*Photon Systems Instruments*).

For the growth with agitation of *Escherichia coli* liquid cultures, two rotary incubators *Infors AG*, *Aerotron* and *Unitron* models were used. The growth on solid medium, without agitation, was carried out in a *Selecta Incubat* 80 L stove.

Sterility conditions

The culture media, the material used to handle the nucleic acids and the solutions that are used for all the material that is in contact with the cultures, were sterilized in a *Selecta* model *75 Presoclave* autoclave. When it was necessary to work under sterile conditions, two laminar flow chambers were used: *Telstar AV-100* and *Telstar BIO-II-A*.

Centrifuges and rotors

Centrifugations were carried out in the following systems:

- Volumes less than 2 mL: in an *Eppendorf* microfuge, models *5415D* and *5415R*, and in a *Beckman Avanti J-25* centrifuge equipped with a *JA-18.1* and *TLA 120.2* rotors.
- Volumes between 2 and 10 mL: in *Mixtasel Selecta* table-top centrifuge and *Optima™ TLX Ultracentrifugue* with rotors *TLA-100.4* and *TLA-120.2*.
- Volumes greater than 10 mL: in a *Beckman Avanti J-25* centrifuge with rotors *JA-20* and *JA-14.*

Spectrophotometric determinations

The absorbance measurements were made in a *Beckman DU-640* spectrophotometer, with capacity for six cuvettes, coupled to a thermostatic bath model *Haake DC1-V* that allowed to control the temperature of the samples when necessary.

The determinations at wavelengths below 340 nm were carried out in quartz cuvettes; above this value, plastic or glass cuvettes were used.

To measure the concentration of nucleic acids samples, a *ND-1000 Spectrophotometer (NanoDrop Technologies)* was used.

pH determination

The pH measurements of the different solutions and buffers used were made with a *CRISON* pH meter model *MicropH2001*, equipped with an electrode from the same manufacturer.

Weight determination

They were performed on *a METTLER PB303 DeltaRange* scale, and on an analytical balance (sensitivity of 0.1 mg) *Sartorius BP1215*.

Agitation of solutions

Solutions that required strong agitation were homogenized in *Heidolph Reax Top* agitators.

For the dissolution of reagents in liquid media, a *Selecta Asincro* magnetic stirrer was used; and when the simultaneous heating of the solution was necessary, a *Selecta Agimatic-N* agitator was used.

Distilled water

The distilled water was obtained with a *Millipore Elix 3* device coupled to a 30 L tank.

A *Millipore Milli-Q* device was used to obtain ultrapure water.

Thermostatization

The thermostatization of the samples was carried out in *Selecta* baths, *Unitronic 320 OR* model and *Univeba*, the first of which also allowed the agitation of the samples. When a more accurate and stable temperature was required, a *Haake* model *DC1-V* bath was used.

The incubations at 30 ºC were carried out in a *Selecta* model 205 stove; incubations at 37 ºC, in a *Selecta Incubat* 80-L stove.

Refrigeration

Samples requiring refrigeration (reagents, solutions of nucleotides or DNA, enzymes and other compounds) were stored at -20 ºC in freezers of standard commercial companies. The solutions of RNA, cells and extracts of *Prochlorococcus* and *Escherichia*

coli were stored at -80 ºC in a *REVCO* freezer. Buffers and other solutions were stored in a refrigerator or in a cold room at 4 ºC.

The flake ice used for the preservation of the samples at 4 ºC while they were outside the refrigeration devices indicated, was obtained by an ITV ice generator model *IQ 135* system.

Power supplies

Routinely, two power supplies of *Bio-Rad*, models *250/2.5* and *PowerPac Basic* were used.

Electrophoresis chambers and transfer system

The separation of protein samples was carried out in *Mini-PROTEAN 3 Cell (Bio-Rad Laboratories)*.

The separation of DNA samples was carried out in 7 x 10 cm (*Mini-Sub Cell GT*) and 15x10 cm (*Wide Mini-Sub Cell GT*) chambers from (*Bio-Rad Laboratories)*.

The protein transfer system used for Western blot was *Trans-Blot SD Semi-Dry Transfer System* (*Bio-Rad Laboratories)*.

RESULTS

Results

Effects of glucose and light on *glcH* expression

The small size of picocyanobacterial genomes (Dufresne et al., 2003; Palenik et al., 2003; Rocap et al., 2003) and the low concentration of sugars in the ocean, in the nanomolar range (Zubkov and Tarran, 2008; Muñoz-Marín et al., 2013), suggested these picocyanobacteria were restricted to producing glucose from photosynthesis. On the other hand, it has been shown that *Prochlorococcus* can take up glucose, showing an increase in the expression of genes involved on its metabolization (Gómez-Baena et al., 2008).

Besides, it has been shown that *glcH* encodes a very high affinity glucose transporter, and that glucose is taken up by natural *Prochlorococcus* populations (Muñoz-Marín et al., 2013). In addition, it was shown that the kinetic parameters of glucose uptake show significant diversity in different *Prochlorococcus* and *Synechococcus* strains (Muñoz-Marín et al., 2017). The phylogeny of *glcH* is very similar to the consensus phylogeny of marine picocyanobacteria, suggesting this gene has been subjected to evolutive selection in the different clades in both *Prochlorococcus* and *Synechococcus*.

All the studies mentioned above suggest that the *glcH* gene encodes a transporter with a very important function for the different populations of picocyanobacteria of the ocean, whose biological function is being tuned by evolutive selection. Therefore, if all the above mentioned is true, it would be expected to find different responses regarding the glucose uptake in different *Prochlorococcus* and *Synechococcus* strains, showing their adaption to different niches. In order to check this hypothesis, six marine cyanobacterial strains were used, to study the regulation of *glcH* gene in laboratory cultures subjected to different glucose concentrations.

We decided to study first the effect of different glucose concentrations on *glcH* expression.

Figure 16 shows the expression of *glcH* in the low-light ecotype *Prochlorococcus* sp. SS120, one of the most studied strains. There was a slight increase (1.5 to 2.5-fold) in *glcH* expression, in samples subjected up to 100 nM glucose, but there was a strong increase (more than 6-fold) in *Prochlorococcus* cultures where a concentration of 1000

nm of glucose was added. The increases were significant for 1 nM (p = 0.0295) and 1000 nM glucose (p = 0.0016).

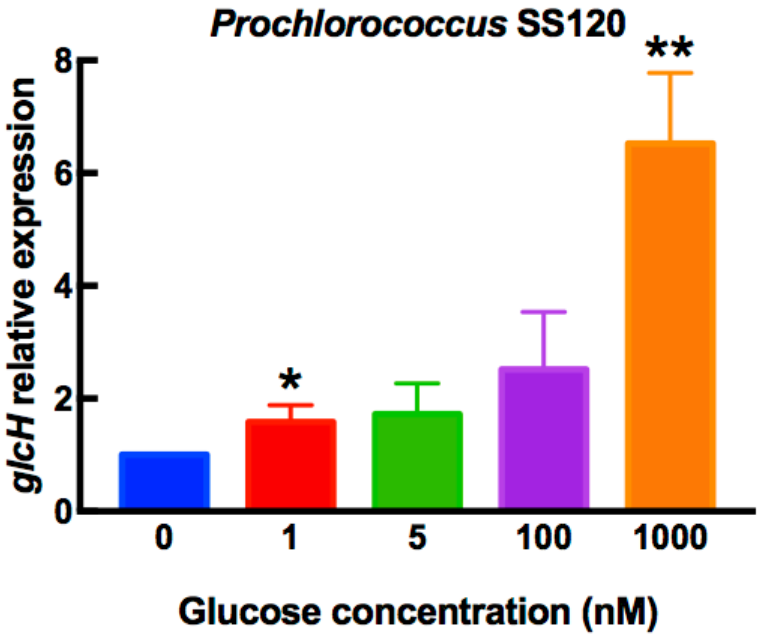


Figure 16. Effect of different glucose concentrations on *glcH* expression in *Prochlorococcus* SS120 strain. *Prochlorococcus* cultures were grown in the light, in a culture room at 24 ºC, supplemented with five different glucose concentrations, and cells harvested after 24 h of treatment. qRT-PCR was used to determine *glcH* expression.

In the case of *Prochlorococcus* sp. MIT9313 (figure 17), the response of *glcH* showed a linear trend along the different concentrations of glucose studied, except for cultures that were treated with 100 nM glucose. The value of *glcH* gene expression observed at 5 nM glucose was highly significant ($p < 0.0001$). Nevertheless, there was no significant difference when comparing the response of strains SS120 vs MIT9313 at 1000 nM glucose ($p = 0.8628$).

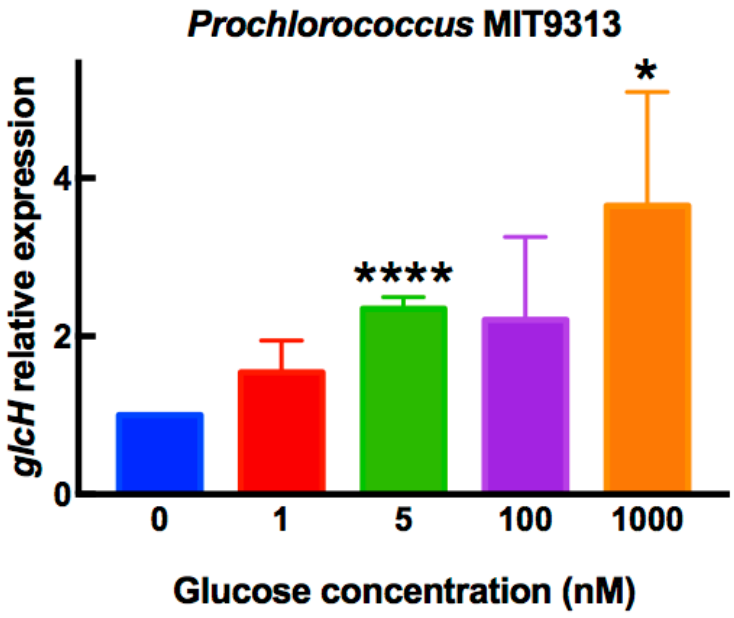


Figure 17. Effect of different glucose concentrations on *glcH* expression in *Prochlorococcus* MIT9313 strain. Culture conditions were the same as those used in figure 16.

In the case of *Prochlorococcus* sp. strain TAK9803-2 *glcH* expression also increased progressively, although the obtained value was lower than in other strains. The maximum values, determined at 1000 nM glucose averaged less than a 3-fold increase, that are half of the values determined for *Prochlorococcus* SS120. Increases were significant at 5 nM ($p = 0.0225$) and highly significant at 1000 nM ($p < 0.0001$).

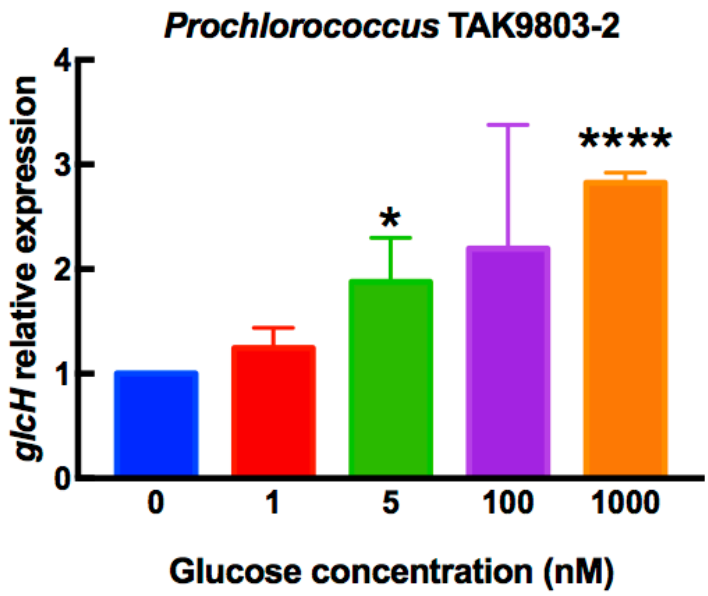


Figure 18. Effect of different glucose concentrations on *glcH* expression in *Prochlorococcus* TAK9803-2 strain. Culture conditions were the same as those used in figure 16.

The response of *glcH* expression in *Prochlorococcus* PCC 9511 was not progressive (figure 19) the values of glucose concentrations of 1, 5 and 100 nM had small effects on *glcH* expression, a marginal increase, with an average value below 1.5-fold with respect to the control culture. Nevertheless, the changes were significant at 5 and 1000 nM glucose ($p = 0.0453$ and 0.0241, respectively). The maximum value found for HL strains was lower than that observed in LL *Prochlorococcus* strains: i.e. 3.65 for MIT9313 and 6.52 for SS120, vs 2.82 for TAK9803-2 and 2.11 for PCC 9511.

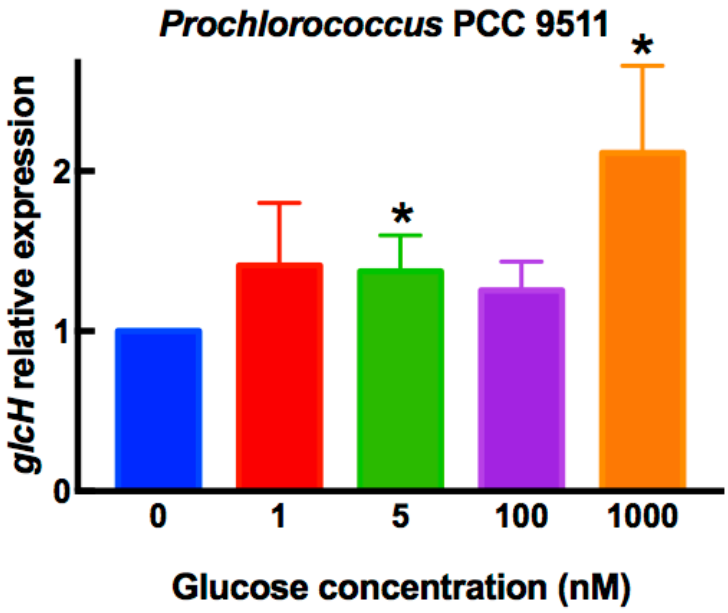


Figure 19. Effect of different glucose concentrations on *glcH* expression in *Prochlorococcus* PCC 9511 strain. Culture conditions were the same as those used in figure 16.

The *Synechococcus* strains had a response different from *Prochlorococcus*: in *Synechococcus* sp. BL107 (figure 20), *glcH* expression showed the maximum value at 5 nM, with a 7-fold increase, whereas other tested concentrations induced lower increases in *glcH*. It is worth noting that this is the only studied cyanobacterial strain where the maximum *glcH* expression was not observed at the maximum tested glucose concentration. Moreover, all observed increases were significant (5 nM glucose, $p = 0.0115$) or highly significant (1, 100 and 1000 nM glucose, $p < 0.0001$).

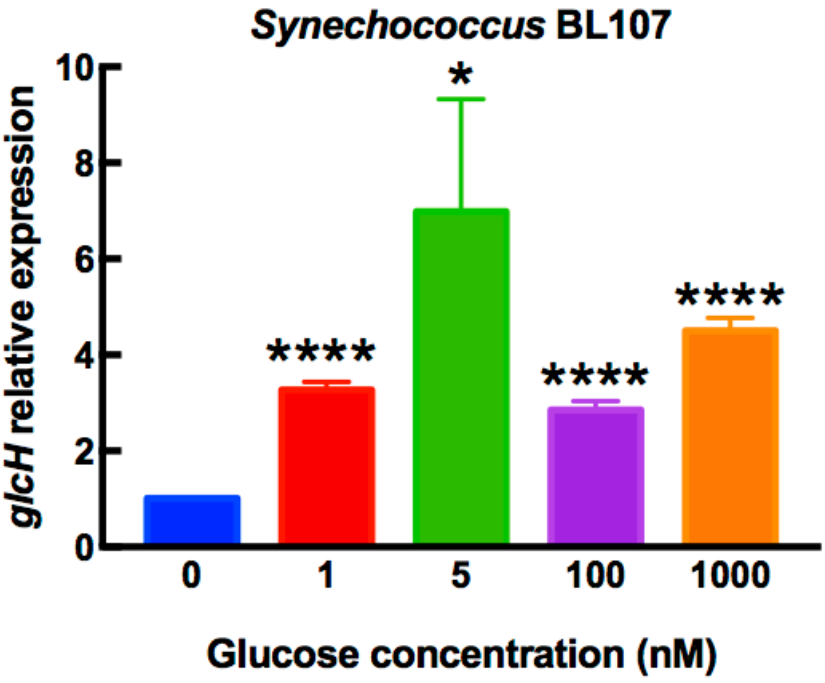


Figure 20. Effect of different glucose concentrations on *glcH* expression in *Synechococcus* BL107 strain. *Synechococcus* cultures were grown in the light, in a culture room at 24 ºC, supplemented with five different glucose concentrations, and cells harvested after 24 h of treatment. qRT-PCR was used to determine *glcH* expression.

On the other hand, *Synechococcus* sp. WH7803 (figure 21) does respond to the addition of glucose progressively, while the overall effect is minor, with very weak increases at all tested glucose concentrations, with the only exception of 1000 nM glucose. In this case, only the value observed at 100 nM was significant ($p = 0.0170$), it shows a different trend that *Synechococcus* BL107 strain.

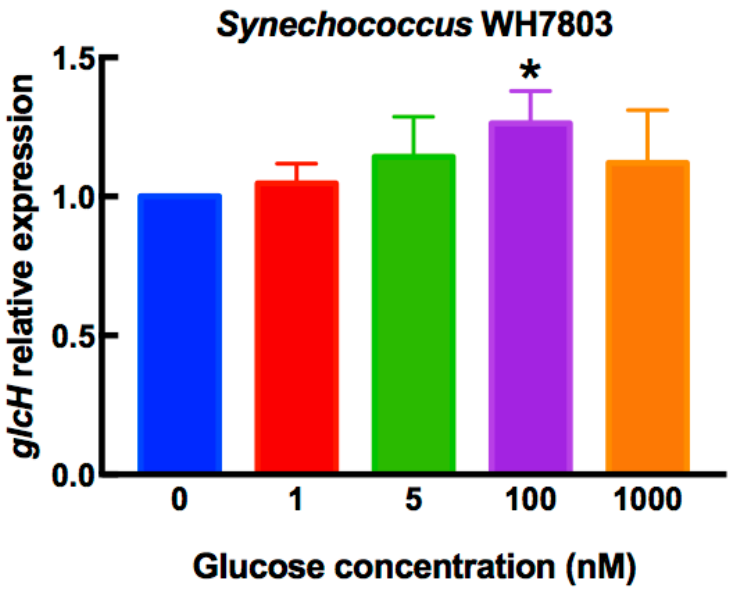


Figure 21. Effect of different glucose concentrations on *glcH* expression in *Synechococcus* WH7803 strain. Culture conditions were the same as those used in figure 20.

A comparative analysis of the results or the six tested cyanobacterial strains shows that the response in *Synechococcus* clearly differs from *Prochlorococcus* and exhibits two almost antagonistic profiles; from the almost no-change of *Synechococcus* WH7803, to the bell shape observed in *Synechococcus* BL107. These results suggest that a more in-depth and detailed study within the *Synechococcus* genus would show a wider diversity in the response of *glcH* expression, in good agreement with the reported phylogenic diversity of this genus.

In these experiments, the transcriptional response of *glcH* to several glucose concentrations in different *Prochlorococcus* and *Synechococcus* strains (Moreno-Cabezuelo et al., 2019) has been tested. In all studied strains *glcH* was expressed in the absence of glucose, and it increased upon glucose addition to the cultures. The response was different depending on the strain; in the way cells responded to the tested glucose concentrations and magnitude.

The results show a diversity of responses depending on the strain, which indicates that the expression of *glcH* is regulated differently depending to the ecotype studied.

This reinforces the importance of glucose uptake for the ecology of marine cyanobacteria and throws light on the relevance of the mixotrophy for these ecologically important cyanobacteria.

Effect of light on *glcH* expression

It has been shown that darkness induces a decrease in the glucose uptake levels in *Prochlorococcus* sp. SS120 (Gómez-Baena et al., 2008). Moreover, it has been shown that the addition of the inhibitors of the photosynthetic electron transport DCMU and DBMIB also inhibited glucose uptake in the same *Prochlorococcus* strain (Muñoz-Marín et al., 2017). For these reasons, it was decided to study the effect of darkness on the expression of *glcH* to assess the possible diversity of responses in different *Prochlorococcus* and *Synechococcus* strains.

Effect of light on *glcH* expression in *Prochlorococcus*

Figure 22 shows the results obtained in different *Prochlorococcus* strains. The value obtained when *glcH* expression was determined in control cultures for each strain (growing under light) received the value of 1. In this way, the first bar in Figure serves for comparison to all strains shown at the right part of the chart. The effect of darkness provoked a strong decrease in *glcH* expression in all *Prochlorococcus* strains. This decrease ranged from a minimum of a significant 60 % (in the case of *Prochlorococcus* sp. SS120; $p = 0.0035$) to an almost complete inhibition of *glcH* expression (97 % decrease in the case of *Prochlorococcus* sp. strain MIT9313, ($p < 0.0001$). These results highlight the lack of similarity in the responses of the two studied low-light adapted strain. The level of expression of the *glcH* gene is found at an intermediate point in the strains TAK9803-2 and PCC 9511, with darkness-promoted decreases of 76 % ($p < 0.0001$) and 88 % in *glcH* expression ($p = 0.0001$), respectively.

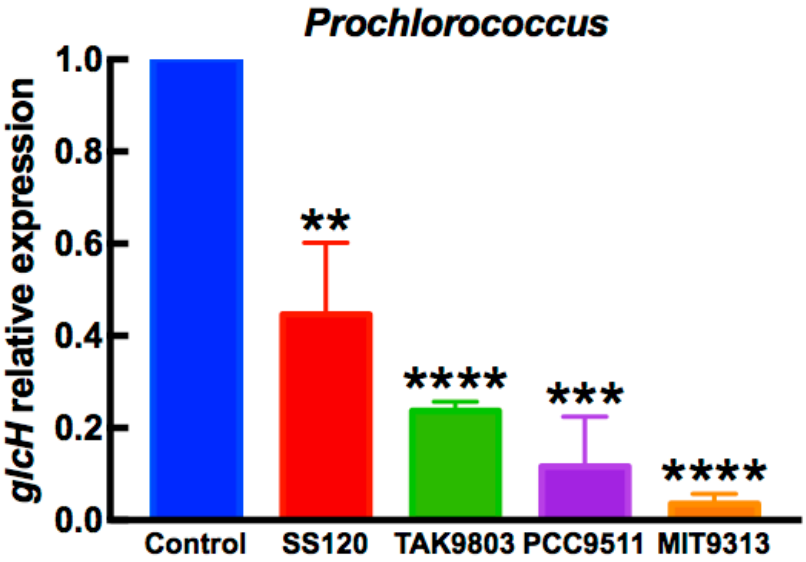


Figure 22. Effect of darkness on *glcH* expression in *Prochlorococcus*.
Prochlorococcus cultures were divided in two aliquots: one of them was kept under standard irradiance used as control and the other was subjected to darkness. After 24 h in these conditions, cells were harvested and used to determine *glcH* expression.

Effect of light on *glcH* expression in *Synechococcus*

The effect of darkness in strains of *Synechococcus* was studied in the same way as in the strains of *Prochlorococcus* (figure 23). We observed an intense effect on the *glcH* gene, whose expression was reduced in a 70% in the case of *Synechococcus* BL107 ($p < 0.0001$), and in a 63 % in the case of *Synechococcus* WH7803 ($p = 0.0001$). These results are similar to those observed for *Prochlorococcus* sp. SS120, and lower compared to those for the other *Prochlorococcus* strains.

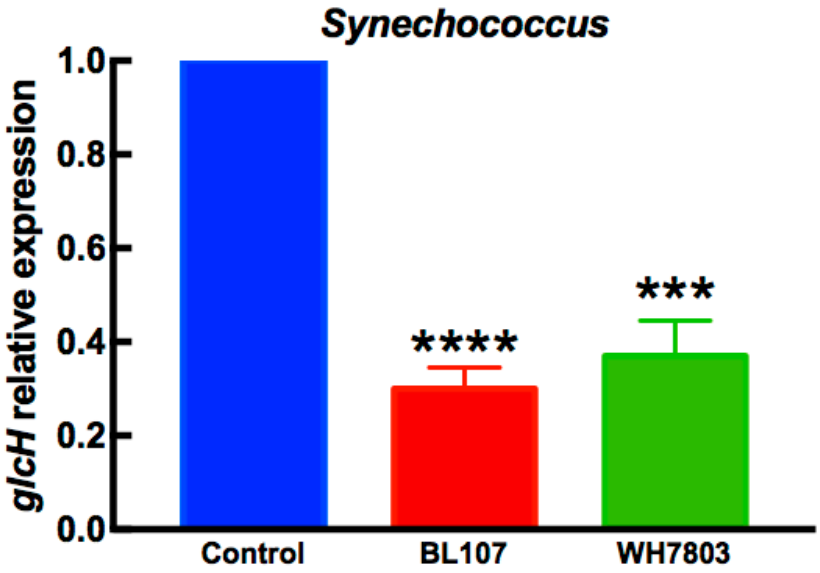


Figure 23. Effect of darkness on *glcH* expression in *Synechococcus*.
Synechococcus cultures were divided in two aliquots: one of them was kept under standard irradiance used as control and the other was subjected to darkness. After 24 h in these conditions, cells were harvested and used to determine *glcH* expression.

Selection of essential amino acid residues of Pro1404 transporter

In order to analyze the role of specific amino acid residues in the glucose uptake carried out by GlcH, we undertook a bioinformatic study to select potential key amino acid residues. We aimed at creating recombinant forms of the GlcH transporter with mutated residues, to study their effects on glucose uptake.

For the selection of essential residues of the *Pro1404* gene product, annotated in the genome of *Prochlorococcus* marinus SS120 as *melB/glcH* (a putative transporter melibiose/sodium symport), an alignment of the amino acid sequence encoded by this gene was made with that of homologous genes in other cyanobacteria, as well as with that of *melB* of *Salmonella typhimurium* from which the three-dimensional structure has been obtained (Ethayathulla et al., 2014). This alignment was performed with ClustalW at the bioinformatics resources portal ExPASy (https://embnet.vital-it.ch/software/ClustalW.html). This allowed to identify which residues are evolutionarily conserved and to select those of greatest interest to carry out the directed mutagenesis. From the study of the MelB transporter of *Salmonella typhimurium* (Ethayathulla et al., 2014) the following residues were considered to be essential: Asp55, Asn58, Asp59, Asp124, Tyr120, Thr121 y Thr373 participate in the union of the cation (sodium) to the transporter. Asp19, Tyr120, Asp124, Trp128, Arg149 and Lys377 participate in the binding of sugar to the transporter. Arg141, Arg295 and Arg363 participate in the formation of "ionic locks", that is, they are important for establishing the different conformations of the transporter, since they are involved in the formation of electrostatic interactions. To carry out the alignment, the homologous sequences in the cyanobacteria shown in table 31 were used, along with the sequence of *Salmonella typhimurium*:

Table 31. Cyanobacterial strains used in the alignment of GlcH/Pro1404 amino acid sequences.

Cyanobacteria	Gene	Homology[1]
Prochlorococcus marinus sp. SS120	*Pro1404*	100 %
Prochlorococcus marinus sp. MED4	*PMM1323*	64.3 %
Prochlorococcus marinus sp. MIT9313	*PMT1398*	63.4 %
Synechococcus sp. WH7803	*SynWH7803_1931*	61.3 %
Synechocystis sp. PCC 6803	*sll1374*	38.8 %

[1]The homology of the gene is considered taking *Prochlorococcus marinus sp.* SS120 as a reference.

The alignment results showed that Asp55, Asn58, Asp59, Tyr120, Thr121, Arg141 and Lys377 are conserved residues in all organisms and Asp124, Arg295 and Arg363 have undergone conservative substitutions in some of the organisms used for the alignment.

Based on the results obtained, two residues were selected to carry out the directed mutagenesis: Asp59 and Arg141, which in *Prochlorococcus marinus sp.* SS120 correspond to the residues Asp52 and Arg134. We outlined a strategy to carry out directed mutagenesis of these two amino acids to Gly, obtaining the mutants D52G and R134G respectively.

Directed mutagenesis of essential residues of Pro1404 transporter

Introduction of the mutation by overlapping PCR

The overlapping PCR (Kanoksilapatham et al., 2007) consists on the use of two rounds of PCR amplification, with an overlapping extension stage between both, using two flanking primers and two partially or totally complementary internal primers designed to generate specific mutations.

In the first round of PCR, each of the mutagenic primers with their respective flanking primer amplify fragments that will then overlap, carrying the mutation at their ends. Finally, the fragments obtained are used in a second round of PCR together with the flanking primers to obtain the complete mutated fragment.

The process is outlined in the following figure.

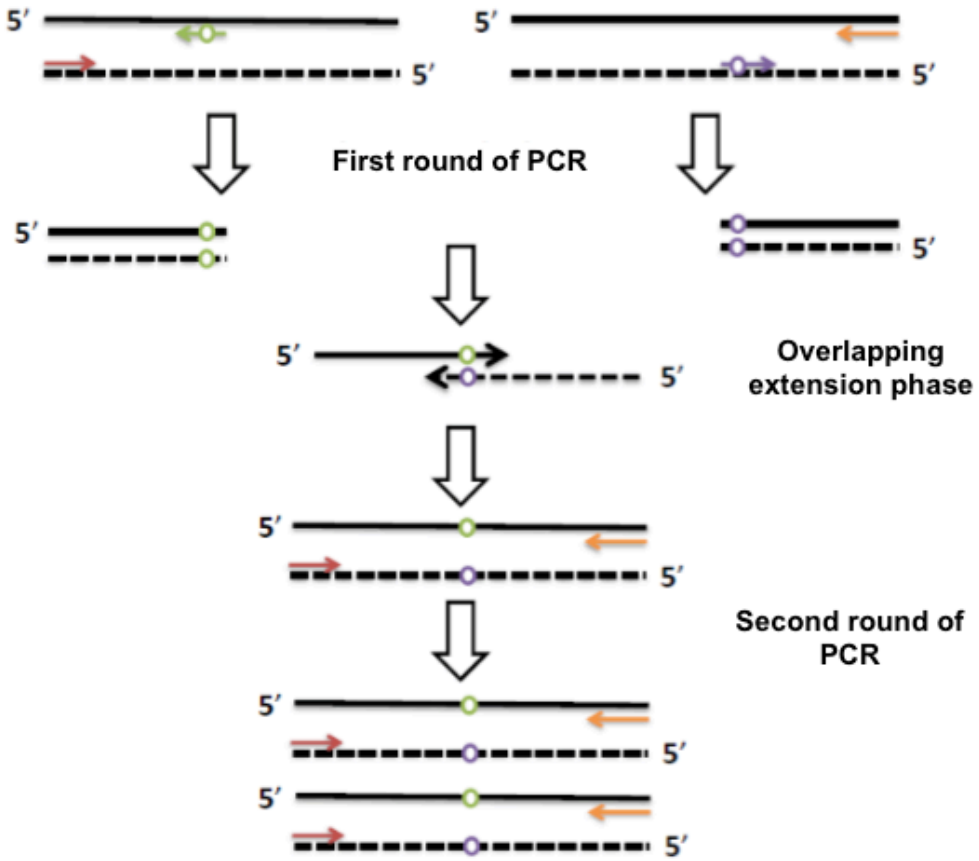


Figure 24. Scheme of overlapping PCR, modified from (Kanoksilapatham et al., 2007).

Table 32. Primers used to perform directed mutagenesis in the *glcH* gene.

Mutant	Primer	Expected fragment size
R134G	Pro1404-3[1]	160 bp
	Pro1404-12[2]	
	Pro1404-13[2]	466 bp
	Pro1404-6[1]	
D52G	CK1-2[2]	334 bp
	Pro1404-14[1]	
	Pro1404-15[1]	1769 bp
	7942_asnS_4R[2]	

[1]Internal primers designed to generate the desired mutations.
[2]Flanking primers.

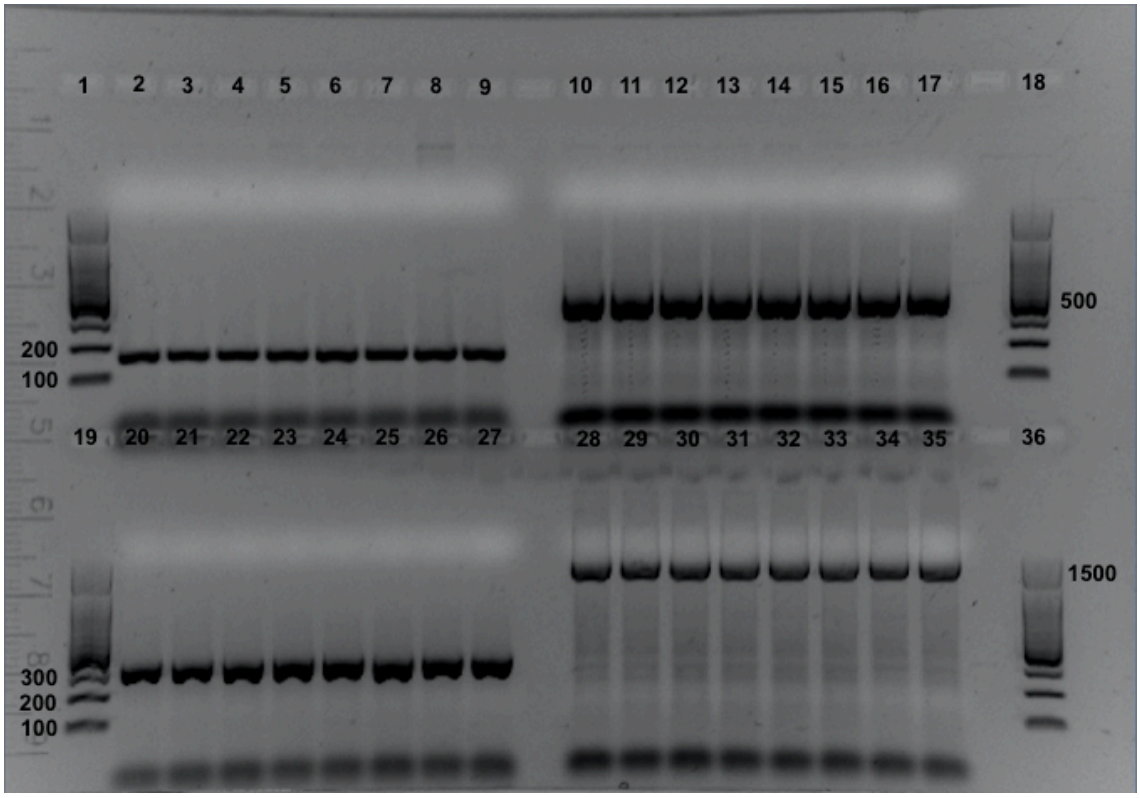


Figure 25. Results obtained in the first round of PCR. Lanes 1, 18, 19 and 36: size markers. Lanes 2 - 9 show the result of PCR amplifications using primers Pro1404-3 / Pro1404-12 at different elongation temperatures. Lanes 10 - 17 show the result of amplifications using primers Pro1404-13/ Pro1404-6. Lanes 20 - 27 show the result of amplifications using primers CK1-2/ Pro1404-14. Lanes 28 - 35 show the result of amplifications using primers Pro1404-15/ 7942_asnS_4R.

The products obtained were used, as indicated above, in a second round of PCR that takes place after the overlapping extension stage, together with the flanking primers, to obtain the complete mutated fragment.

In order to obtain a greater amount of DNA, a PCR was performed with twice the volume. (figure 26).

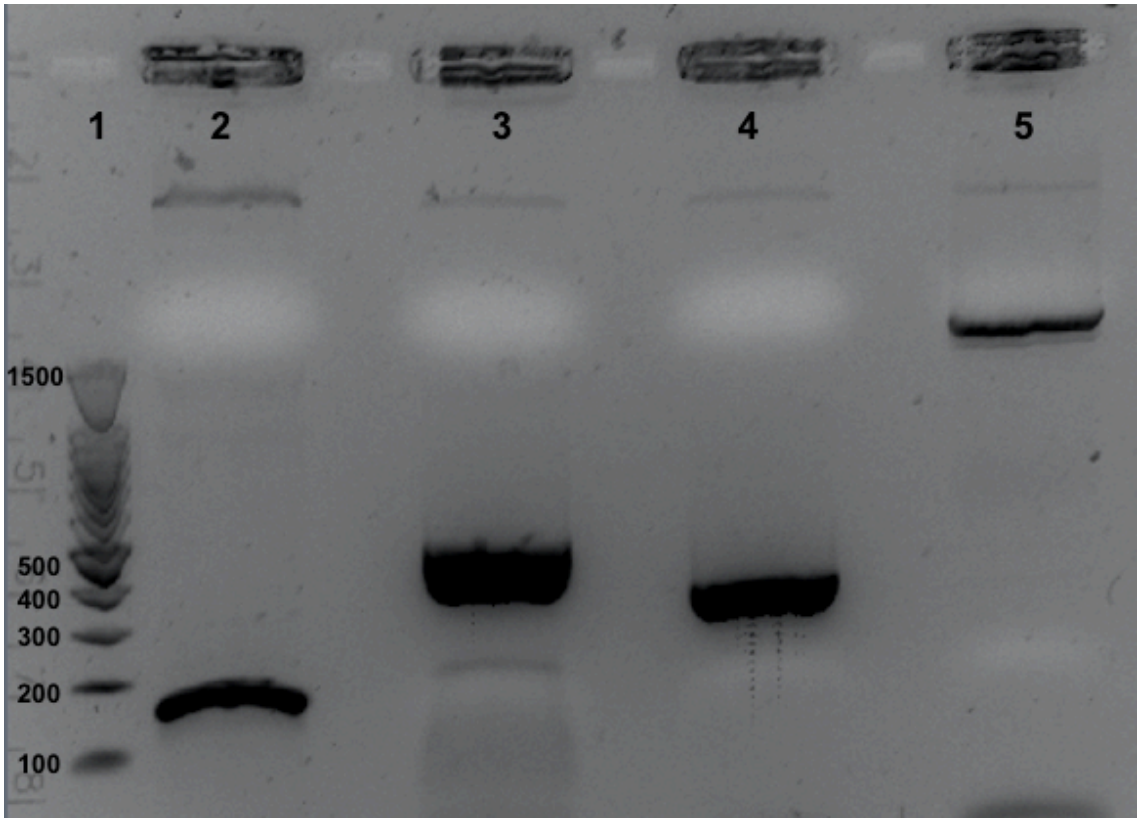


Figure 26. Results obtained in the first round of PCR. Lane 1: size markers. Lanes 2 - 5: fragments PCR amplified with primers Pro1404-3 / Pro1404-12, Pro1404-13 / Pro1404-6, CK1-2 / Pro1404-14 and Pro1404-15 / 7942_asnS_4R respectively. From now on these fragments will be named from 1 to 4, respectively.

The products obtained were used, as indicated above, in a second round of PCR that takes place after the overlapping extension stage, together with the flanking primers, to obtain the complete mutated fragment. The results obtained showed the product of the union of fragments 1 and 2, from now called AMP-1, whose size coincides with that expected (609 bp) (figure 27). In addition, the product of the binding of fragments 3 and 4 (AMP-2) was obtained and corresponds to the expected size (1769 bp) (figure 28).

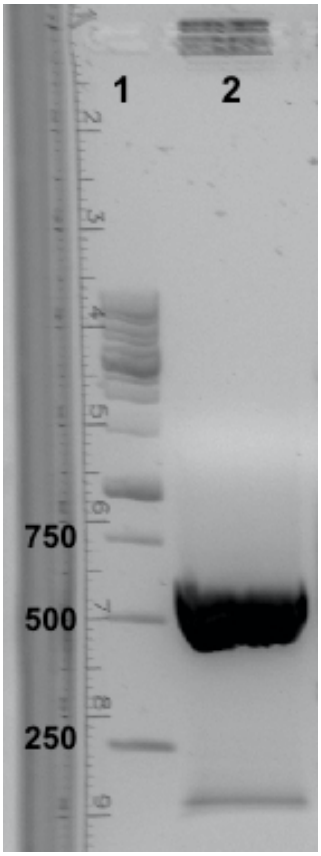


Figure 27. Results obtained in the overlapping extension stage and second round PCR of the AMP-1 fragment after optimization of the process. Lane 1: size markers. Lane 2: AMP-1, result of the union of fragments 1 and 2.

To optimize the process, DMSO was added to the PCR mixture at a final concentration of 3% to fragments 1 and 2, and also to fragments 3 and 4, and subjected to the overlapping extension phase followed by the second PCR round. DMSO contributes to the decrease of the DNA Tm, that is, to the decrease in the temperature at which half the amount of DNA has been denatured, thus increasing the specificity and fidelity of the PCR (Simonovic et al., 2012).

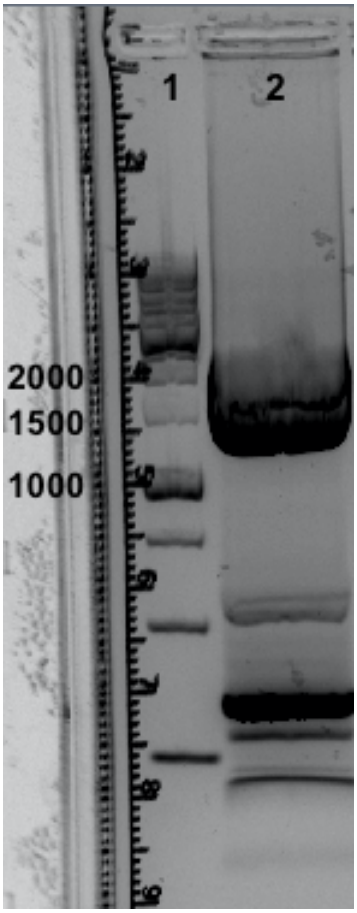


Figure 28. Results obtained in the overlapping extension stage and second round PCR of the AMP-2 fragment after the optimization of the process. Lane 1: size markers. Lane 2: AMP-1, result of the union of fragments 3 and 4.

At this point we had, therefore, the amplified AMP-1, which should contain the R134G mutation, and the amplified AMP-2, with D52G mutation.

Cloning of the amplified fragments in a cloning vector

The products obtained in the overlapping PCR were cloned in the *pSpark II vector*. For this, a ligation of the amplified fragments (AMP-1 and AMP-2) with the cloning vector was carried out, and with this mixture competent *Escherichia coli* HB101 cells were transformed. The plasmid resulting from the ligation of AMP-1 with the vector *pSpark II* was called pcJA1, and that of AMP-2 with the same vector was called pcJA3. In both cases, two different plasmids could be obtained in each of the ligations, which were called a and b, depending on the orientation in which the insert had been introduced into the vector.

Verification of the plasmid constructs by DNA sequencing

Sequencing, in addition to confirm definitively the presence of the insert and its orientation within the plasmids, pcJA1 and pcJA3, allowed to verify the presence of the introduced mutation and rule out other possible mutations derived from the PCR amplification. Sequencing was carried out using primers T7 and Sp6 for the AMP-1 fragment. The coincidence of the alignment of the theoretical sequence of AMP-1 with that obtained in the sequencing was 100%.

In the case of the AMP-2 fragment, in addition to the primers T7 and Sp6, the primers Pro1404-9 and Pro1404-11 were also used, obtaining a coincidence of the alignment of the theoretical sequence of AMP-2 with that obtained in the sequencing of the 100 %.

Cloning of the mutated versions of Pro1404

Once the presence and orientation of each of the inserts in the *pSpark II* cloning vector was verified, as well as the presence of the mutation within the insert, a preparative digestion of the plasmids pcJA1a and pcJA3a and the pcMM3 vector was carried out, which includes the *Pro1404* gene under the control of the kanamycin resistance cassette promoter C.K3, with the restriction endonucleases that are detailed in the table 33 and are shown in figure 29.

Table 33. Restriction enzymes used to digest the plasmids and expected size of the digestion products.

Plasmid	Enzyme	Expected size of the fragment
pcJA1a	*SpeI*	3043 bp 503 bp 64 bp
pcMM3	*SpeI*	7948 bp 503 bp
pcJA3a	*NdeI, XhoI*	3140 bp 1868 bp 72 bp
pcMM3	*NdeI, Xhol*	6583 bp 1868 bp

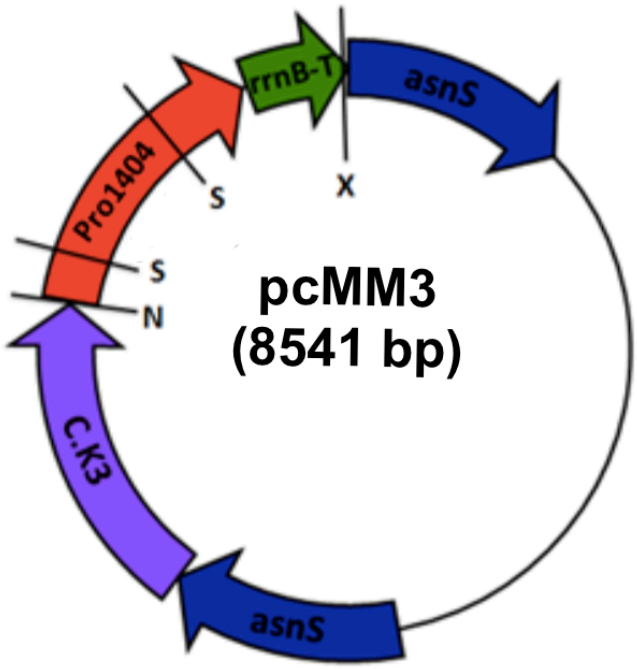
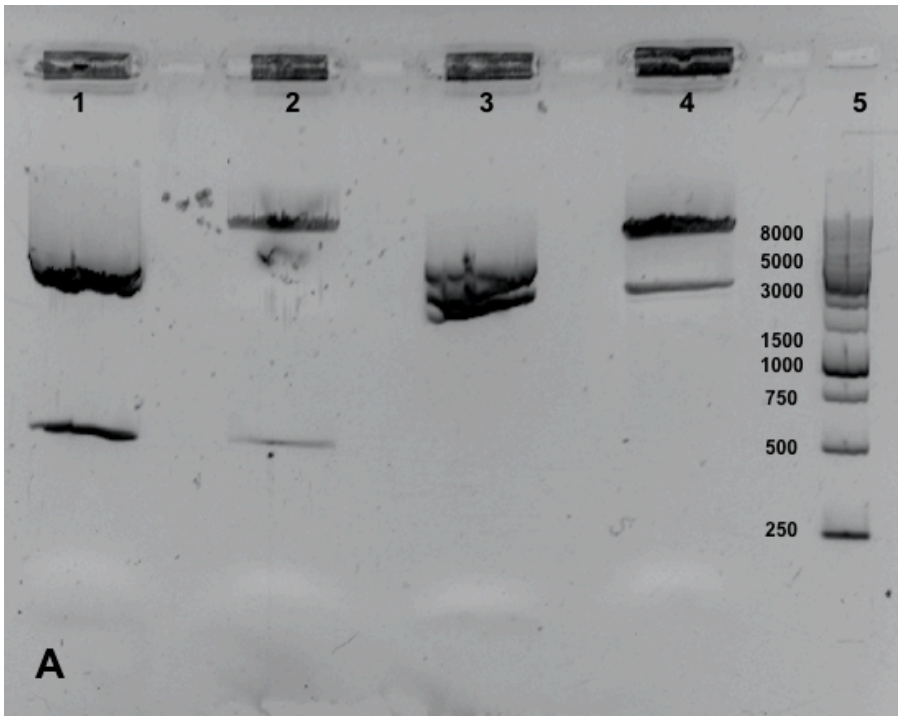


Figure 29. Scheme with the restriction endonuclease cleavage sites used in preparative digestions. *Pro1404*: *glcH* gene, encoding a high affinity glucose transporter. *rrnB*-T: *rrnB* transcriptional terminator. *asnS*: gene encoding asparagynyl-tRNA synthetase. C.K3: Kanamycin-resistance cassete with a strong promoter and no transcriptional terminator. N: *NdeI* site. X: *XhoI* site. S: *SpeI* site.

The process of restriction enzyme digestion is shown in figure 30:



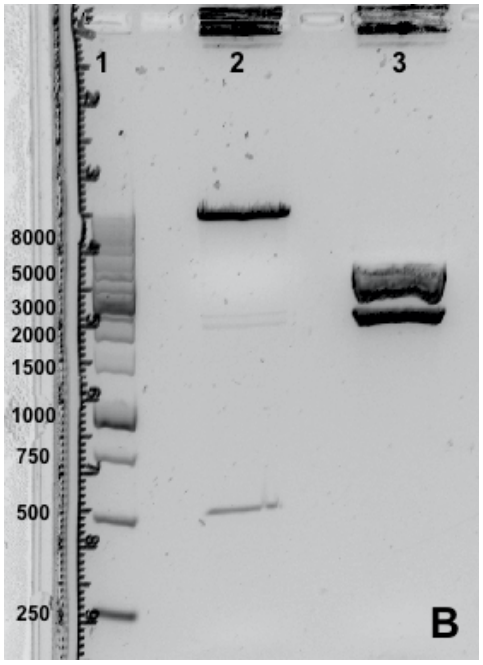


Figure 30A. Results obtained after preparative digestions of plasmids. Lane 1: pcJA1a digested with *SpeI*. Lane 2: pcMM3 digested with *SpeI*. Lane 3: pcJA3a digested *NdeI* and *XhoI*. Lane 4: pcMM3 digested with *NdeI* and *XhoI*. Well 5: Size markers.
Figure 30B. The result of pcJA3a digested *NdeI* and *XhoI* was repeated. In addition, the digestion of plasmid pcMM3 was repeated with the *SpeI* restriction enzyme to obtain a greater amount of DNA. Lane 1: size markers. Lane 2: pcMM3 digested with *SpeI*. Lane 3: pcJA3a digested *NdeI* and *XhoI*.

The products resulting from the digestion of pcJA1a with *SpeI* and pcJA3a with *NdeI* and *XhoI*, which contained the mutation, were cloned into the pcMM3 vector. To do this, prior to ligation, 500 ng of the pcMM3 vector digested with a single restriction endonuclease (pcMM3 x *SpeI*) was dephosphorylated to avoid a high religation frequency. The remaining amount of pcMM3 x *SpeI* was not dephosphorylated, acting as a control. After ligation of the inserts from pcJA1a and pcJA3a with the larger digested fragment obtained from pcMM3, resulting in the plasmids which we called pcJA2 and pcJA4, the ligation mixtures were used to transform the competent cells of *Escherichia coli* HB101.

In the case of pcJA2, two different plasmids could be obtained, which were called a and b, depending on the orientation in which the insert had been introduced into the vector. In the case of plasmid pcJA4, there was only one possibility, since digestion was carried out with two restriction endonucleases instead of one.

Plasmid pcJA2

Since the presence of the introduced mutation was already verified after sequencing of pcJA1a, the aim of sequencing in this case was limited to confirmation of the presence of the insert and its orientation within the plasmid pcJA2. Sequencing was carried out using the Pro1404-6 primer. The alignment of the theoretical sequence of this region with that obtained in the sequencing was 100%.

Plasmid pcJA4

Since the presence of the introduced mutation was already verified after the sequencing of pcJA3a and that, moreover, the insert can only be introduced in an orientation in the vector, the aim of the sequencing in this case was limited to confirming the presence of the insert sequencing was carried out using the Pro1404-9 primer.

The coincidence of the alignment of the theoretical sequence of this region of pcJA4 with that obtained in the sequencing was 100%.

Transformation of *Synechococcus elongatus* PCC 7942 with the mutated versions of Pro1404

After checking the presence of the respective inserts in pcJA2 and pcJA4, as well as the orientation thereof in the case of pcJA2, the transformation of *Synechococcus elongatus* PCC 7942 was carried out as described in the Materials and Methods section, obtaining the mutants JA2 and JA4, respectively. Both constructs were introduced by recombination with the *asnS* gene (encodes asparaginyl-tRNA synthetase) in the chromosome of *Synechococcus elongatus* PCC 7942. It is worth noting that the strategy of cloning and expressing the *glcH* gene within *asnS* in *S. elongatus* PCC 7942 had been successfully previously carried out, given that *asnS* is a dispensable gene in this cyanobacterial strain (Muñoz-Marín et al., 2013).

Verification of the correct construction of the JA2 and JA4 mutants of *Synechococcus elongatus* PCC 7942

Five kanamycin-resistant colonies were isolated from the filter corresponding to the transformation of *Synechococcus elongatus* PCC 7942 with the plasmids pcJA2 and pcJA4, and the insertion of the mutated version of *Pro1404* in the genome of this cyanobacterium was confirmed by PCR. For this, the primers that are detailed in the table 34 were used and are shown in figure 31. Five different colonies were used to carry out the PCR process. 3 of them were found to correspond to the mutant pcJA2, and 2 to the mutant pcJA4. In addition, a colony of untransformed *Synechococcus elongatus* PCC 7942 was used in PCR as a control.

Table 34. Primers used to verify the identity of *Synechococcus elongatus* mutants.

Primers	Expected size of the amplified in WT	Expected size of the amplified mutants
7942-asnS-6	Without amplified	1357 bp
CK3-5		
7942-asnS-4F	64 bp	Too big to be able to visualize it
7942-asnS-4R		

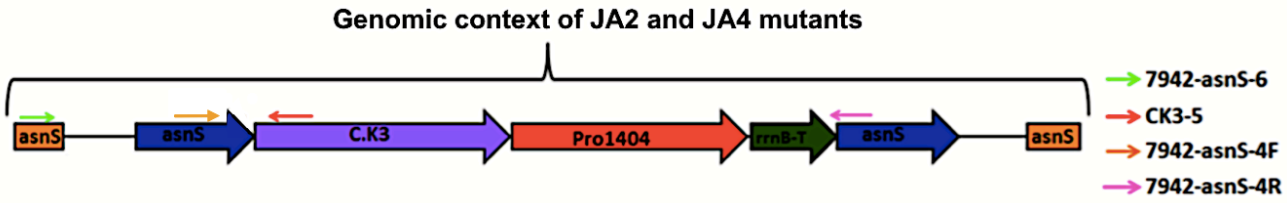


Figure 31. Primers used to check the identity of *Synechococcus elongatus* PCC 7942 mutants.

The result showed that some of the selected colonies have undergone the recombination process, incorporating the mutated version of *glcH* under the control of the resistance cassette C.K3

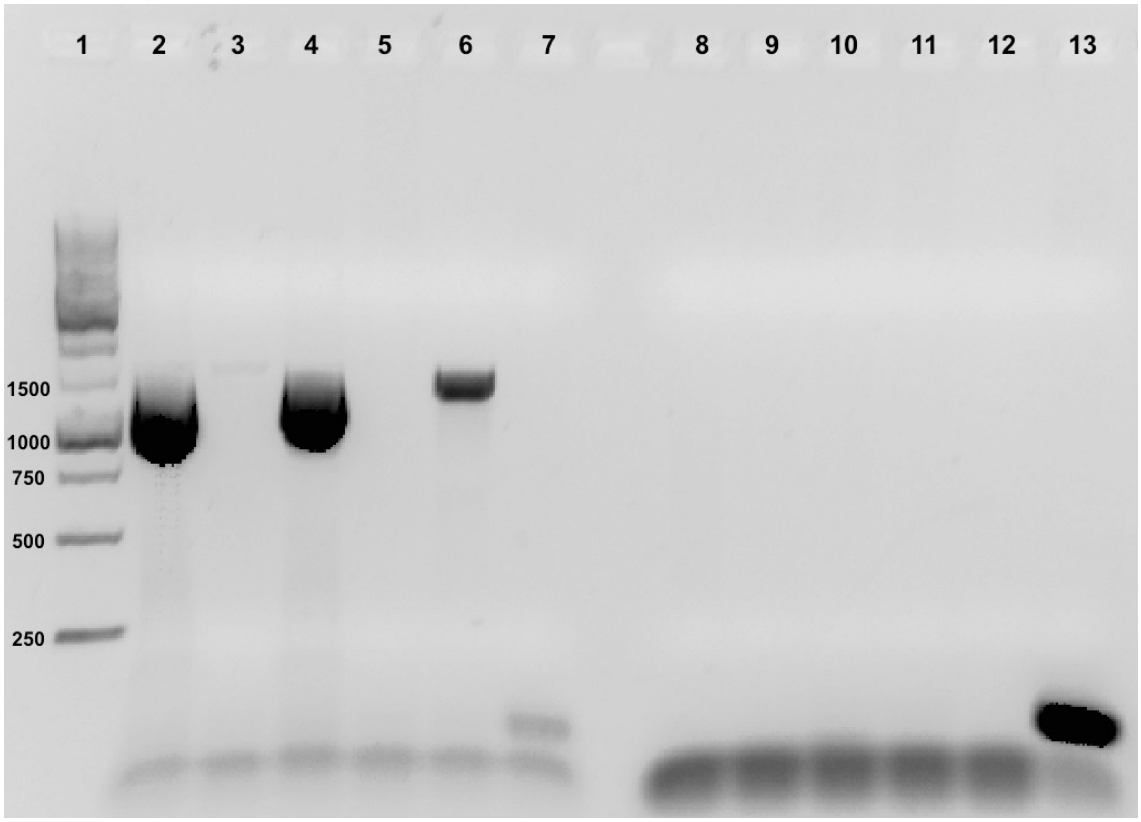


Figure 32. PCR verification of the construction of *Prochlorococcus* sp. SS120 *glcH* mutants. Genomic DNA was isolated from the different colonies and used to carry out the PCR with 2 pairs of primers for confirmation, as indicated:
Lane 1: size markers. Lanes 2, 3 and 4: JA2 mutant amplified with 7942-asnS-6/CK3-5. Lanes 5 and 6: JA4 mutant amplified with 7942-asnS-6/CK3-5. Lane 7: wild type amplified with 7942-asnS-6/CK3-5. Lanes 8, 9 and 10: JA2 mutant amplified with 7942-asnS-4F/7942-asnS-4R. Lanes 11 and 12: mutant JA4 amplified with 7942-asnS-4F/7942-asnS-4R. Lane 13: wild type amplified with 7942-asnS-4F/7942-asnS-4R.

The amplifications confirmed the identity of the colonies of both mutants. The absence of band in lane 7 is due to the fact that the CK3-5 primer of the kanamycin resistance cassette has no hybridation region in the wild type chromosome, whereas in the case of the colonies of the mutants, which do present the resistance cassette to kanamycin, amplification with 7942-asnS-6 / CK3-5 results in a band of 1357 bp.

The wild type amplification shows, however, a 64 bp band (lane 13) resulting from the amplification with 7942-asnS-4F / 7942-asnS-4R. This is because there is no insert within the *asnS* gene in the genome of *Synechococcus elongatus* PCC 7942.

In the colonies of the mutants, which do present the insert, the fragment resulting from the amplification with 7942-asnS-4F / 7942-asnS-4R would be 6006 bp, too large to be visualized with the electrophoresis protocol that had been established.

Therefore, the results shown in lanes 2 and 4 confirm the amplification of JA2, and those shown in lane 6 confirm the amplification of JA4.

Hence the genome of these two *Synechococcus elongatus* PCC 7942 mutants include mutated versions of the *glcH* gene from *Prochlorococcus marinus* SS120. Indeed, the results show that directed-mutagenesis of the selected essential residues of GlcH, Asp52 and Arg134, have been changed to JA2 and JA4 mutants, respectively. In preliminary studies of glucose uptake, the results obtained were confusing. Therefore, the capability of glucose uptake in these mutants will be studied in future projects.

Overexpression of the glucose transporter GlcH from *Prochlorococcus* sp. SS120 in *E. coli*

Construct design

Membrane proteins play a key role in many fundamental cellular processes such as transport of nutrients, sensing of environmental signals and energy transduction. Despite their importance, structural and functional characterization of membrane proteins still remains a challenge, partially due to the difficulties in recombinant expression and purification (Ma et al., 2013).

Given the importance of the GlcH glucose transporter and its potential for mixotrophy in marine picocyanobacterial populations, we decided to overexpress this protein with two goals: first, to develop polyclonal antibodies in order to study changes in the production of this transporter under different physiological conditions; and second, to obtain enough protein to develop structural studies, with the final goal of determining its structure at high resolution by using crystallographic techniques.

With this objective, we started a collaboration with Dr. Margarida Archer, head of the Membrane Protein Crystallography Laboratory (Instituto de Tecnologia Quimica e

Biologica, Antonio Xavier Universidade Nova de Lisboa, Oeiras, Portugal), where I did a stay of three months focused on the GlcH overexpression in *E. coli.*

In the frame of this collaboration, it was decided to use pOPIN vectors to overexpress GlcH. The OPPF (Oxford Protein Production Facility, Research Complex at Harwell, Rutherford Appleton Laboratory, Oxford, United Kingdom) has developed a suite of expression vectors (pOPIN) for high-throughput cloning of protein constructs. The pOPIN vectors utilise multiple promoter systems to allow for protein production in all major expression systems without the need to make multiple, host- specific vectors for each construct. The vectors use the pUC origin of replication to obtain high yields of plasmid from *E. coli*, as well as the cytomegalovirus (CMV) enhancer, chicken β-actin promoter and rabbit β-globin poly-A site to increase the protein transcription levels. All of the pOPIN vectors include a cleavable His-tag and offer a variety of fusion proteins to facilitate protein expression and purification.

One construct was designed to be cloned into two types of pOPIN vectors suitable for the overexpression of membrane proteins. The first type of vector used was pOPINEneo-3C-GFP (figure 33) and the second pOPINEneo-3C-2STREP (figure 34).

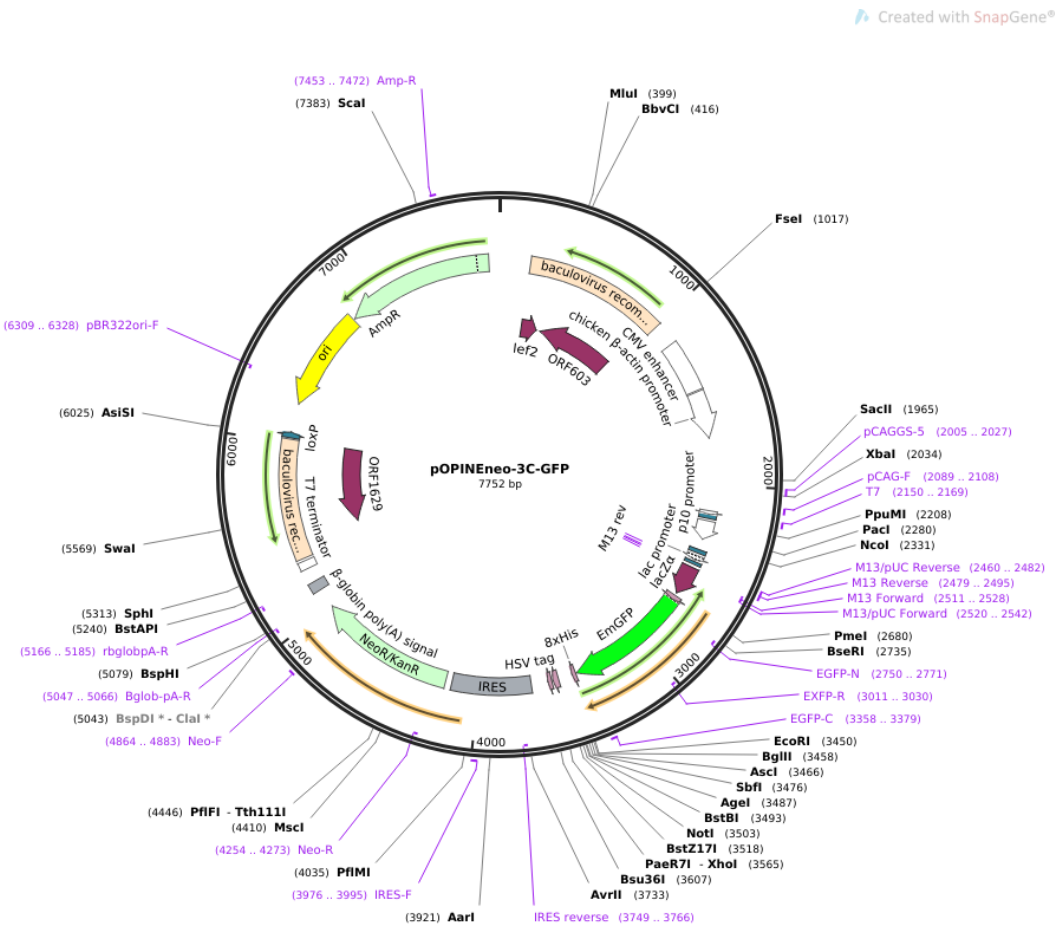


Figure 33. Map of vector used in *Pro1404* expression test. Vector map of pOPINEneo-3C-GFP, highlighting the characteristic features of this vector such as the promoter and enhancer elements that help to increase levels of protein expression and transcription in membrane proteins.

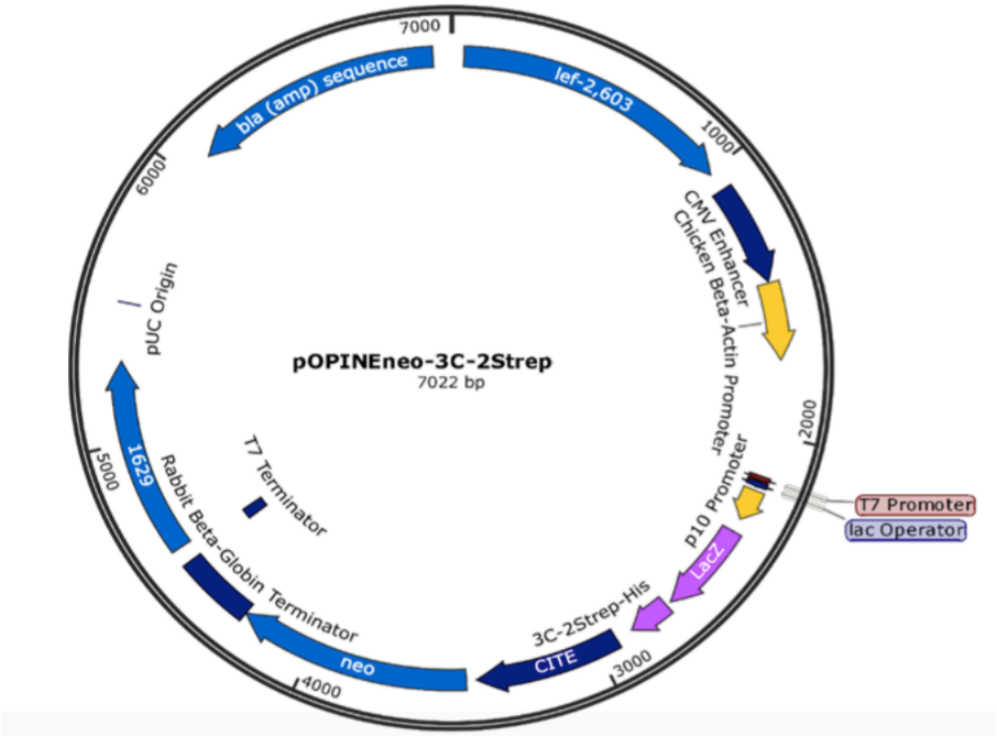


Figure 34. Maps of vector used in *Pro1404* expression test. Vector map of pOPINEneo-3C-2STREP, highlighting the characteristic features of this vector such as the promoter and enhancer elements that help to increase levels of protein expression and transcription in membrane proteins.

Each vector was used to express the *Pro1404* gene of *Prochlorococcus* sp. SS120.

To do this, primers were designed to amplify the complete *Pro1404* gene from *Prochlorococcus* sp. SS120, with a *PmeI* site at both sides of the gene (Pro1404-17:gtttaaacATGCTTTCCTATGGATTA and Pro1404-18: gtttaaacccTAAGTTGTTTATCTGGTA)

Prochlorococcus sp. SS120 genomic DNA was used to carry out the amplification of the gene. As a result, a fragment of 1374 base pairs was obtained, this fragment was called pcJA5 (figure 35).

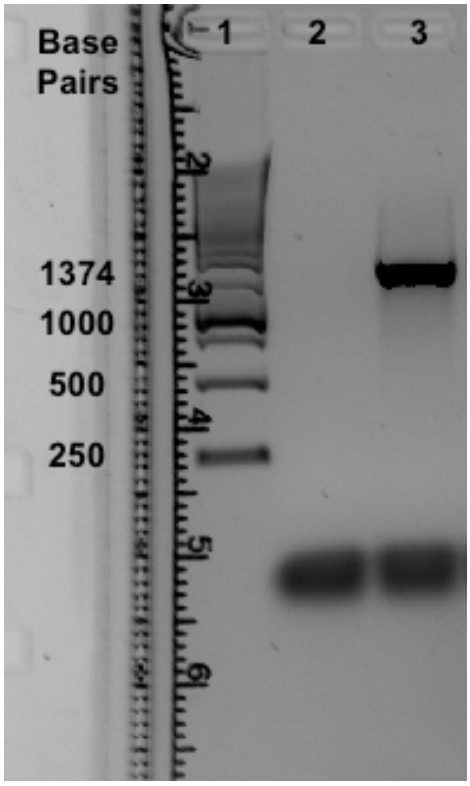


Figure 35. Amplification of *Pro1404* gene with *PmeI* cutting site. Lane 1: molecular weight marker. Lane 2: Control negative (to check that there are no non-specific amplifications). Lane 3: Amplification of pcJA5 (*Pro1404* gene with *PmeI*).

The amplified product was cloned in the plasmid *pSpark*. Sequencing of the plasmid showed that the product was correctly cloned. The resulting plasmid, named pcJA5 was digested with the restriction enzyme *PmeI*. (figure 36).

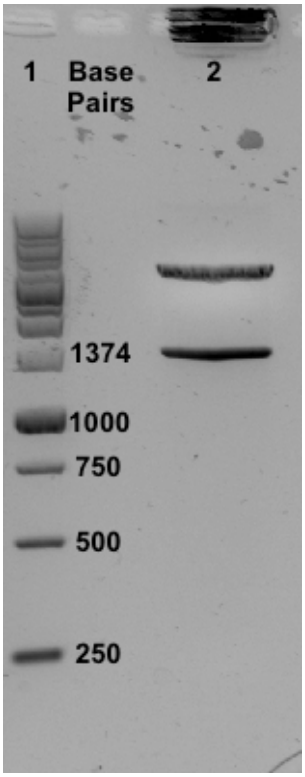


Figure 36. Digestion of pcJA5 plasmid with the restriction enzyme *PmeI*. Lane 1: Size markers. Lane 2: pcJA5 plasmid digested with *PmeI*.

Subsequently the pOPINEneo-3C-GFP and pOPINEneo-3C-2STREP cloning vectors, were digested with the same restriction enzyme (figure 37).

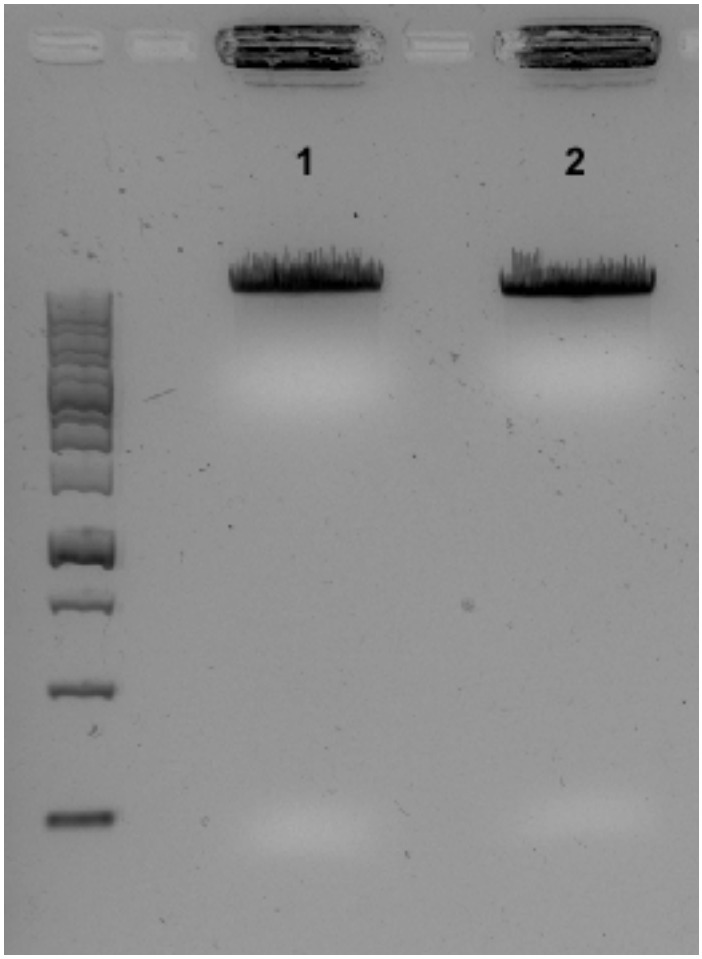


Figure 37. Digestion of pOPIN vectors with *PmeI*. Lane 1. pOPINEneo-3C-GFP digested with *PmeI.* Lane 2. pOPINEneo-3C-2STREP digested with *PmeI.*

The pOPINEneo-3C-GFP and pOPINEneo-3C-2STREP cloning vectors digested with a single restriction enzyme were dephosphorylated, to decrease its religation frequency, and purified. To do this, they were treated with calf intestine alkaline phosphatase *(CIAP)* from *Roche*, as described in Materials and Methods.

Subsequently, the ligation of the insert (*Pro1404*) was carried out with each of the vectors, that is, with pOPINEneo-3C-GFP and pOPINEneo-3C-2STREP, and the ligation products were used to transform *E. coli* cells for generation of high amount of plasmid DNA. For each recombinant strain, two bacterial colonies were tested for the presence of inserted plasmid. The samples were sequenced, and it was found that the constructed plasmids did not have mutations.

Small-scale expression test in *E. coli* cells

To carry out the optimization of the *Pro1404* transporter expression, the plasmids constructed in the previous section; pcJA6A (pOPINEneo-3C-GFP+Pro1404, with a molecular weight of 77.6 kDa) and pcJA7A (pOPINEneo-3C-2STREP, with a molecular weight of 54.6 kDa), were transformed into several strains of *E. coli,* such as *BL21(DE3)*, *BL21-GOLD(DE3)*, *BL21(DE3) PLYSS*, *C41(DE3)* and *C43(DE3)*, and the expression of the studied protein was checked by Western blot.

Standard conditions described in the Materials and Methods section were established, but plasmid pcJA7A showed no expression in any of the transformed strains of *E. coli.*

In the case of the plasmid pcJA6A, it showed expression when it was transformed in the *E. coli* BL21 DE3 GOLD strain using growth temperatures of 30 and 37 °C. However, the size obtained for the expressed protein when it was checked by Western blot did not correspond to the expected size (figure 38).

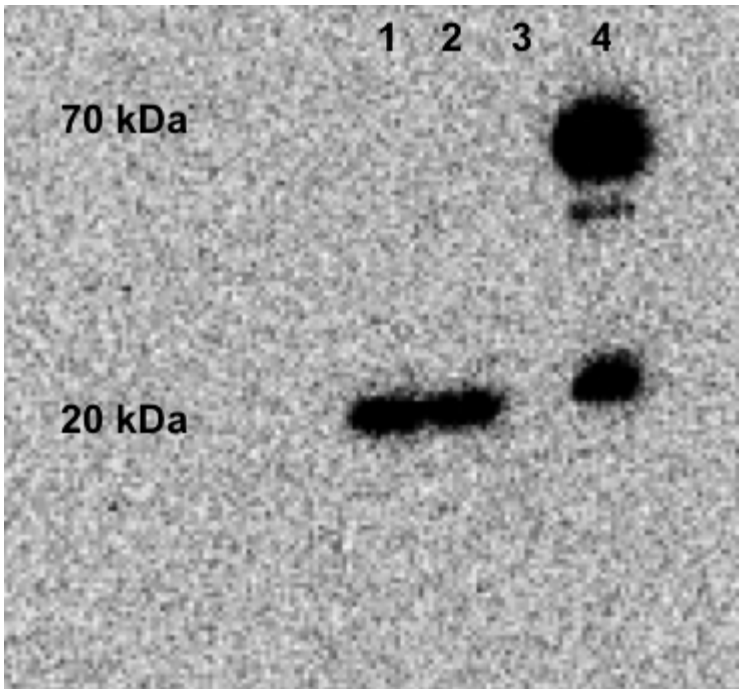


Figure 38. GlcH expression in the pcJA6A plasmid at different growth temperatures.
Lane 1. pcJA6A grown at 37 ºC. Lane 2. pcJA6A grown at 30 ºC. Lane 3. pcJA6A grown at 20 ºC. Lane 4. Hsp 70 protein (positive control).

The protein sizes observed in the Western blot were ca. 20 kDa, corresponding to the GFP (green fluorescent protein), suggesting the occurrence of proteolysis in the samples. We tried to optimize the expression with this plasmid, but it always showed the same profile.

Therefore, an alternative was sought to avoid these problems and obtain the correct expression of GlcH; a different, previously constructed plasmid was used: pcAM8, consisting of a *pET-15b* vector where the *Pro1404* gene had been cloned (Muñoz-Marín et al., 2013).

Surprisingly, this plasmid transformed into *E. coli* Rosetta (DE3) pLysS strain showed a good level of expression. The expected size of the expressed protein was 50 kDa, and that observed in the Western blot was 37 kDa. This value is valid, since migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that does not correlate with molecular weights, called ‘‘gel shifting’’, appears to be common for membrane proteins. It has been shown that the helix-loop-helix “hairpins” migrate at rates of 10% to 30% slower than proteins with their actual formula weights on SDS-PAGE prepared with 3.4–10% SDS (Rath et al., 2009).

Therefore, the plasmid pcAM8 transformed into *E. coli* Rosetta (DE3) pLysS strain provided a correct way to carry out large scale studies in order to overexpress the Pro1404 glucose transporter.

Large-scale expression test in *E. coli* Rosetta (DE3) pLysS strain

E. coli Rosetta expressing the pcAM8 plasmid was grown in LB medium at 37 ºC. When the culture was in exponential phase, plasmid expression was induced with 0.5 mM IPTG, and cells were harvested 4 h after induction. The obtained cell extracts (including supernatans and pellet) were used to optimize solubilization of the GlcH protein by using different detergents. The different fractions of the *Pro1404* transporter were analyzed by Western blot (figure 39).

Table 35. Solubilization test of the membrane fraction of Pro1404-Western Blot.

Lane	Sample	Volume (µL)
1	Marker PPP Dual Color	3
2	Lysate	5
3	Pellet	5
4	Supernatant (ultracentrifugation)	5
5	Membrane fraction	5
6	Triton X 100 (soluble fraction)	5
7	Triton X 100 (pellet fraction)	5
8	DDM (soluble fraction)	5
9	DDM (pellet fraction)	5
10	DM (soluble fraction)	5
11	DM (pellet fraction)	5
12	Positive control- HSP70	1 µg

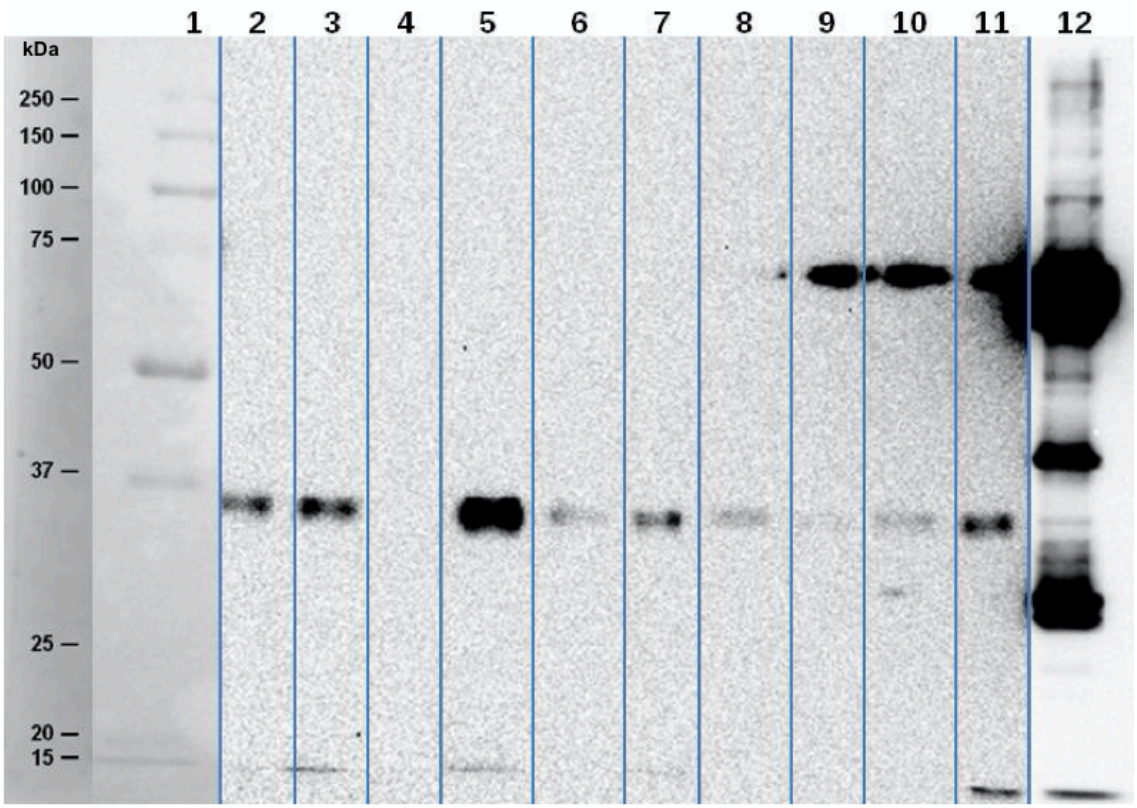


Figure 39. Solubilization test of the membrane fraction of Pro1404-Western Blot.
Membranes were solubilized in 1% (V/V) of detergent. Previous fractions of the cell lysis and membrane separation were also tested in this gel. As a positive control 1 µg of Hsp70 was used. The lysate fraction was obtained after breaking the cells with the French press, as described in the materials and methods section. The pellet fraction was obtained by centrifuging the fraction of the lysate.

The solubilization of membrane proteins is a process by which the proteins and lipids that are held together in native membranes are properly dissociated in a buffered detergent solution. The controlled dissociation of the membrane results in the formation of small protein and lipid clusters. Effective solubilization and purification of membrane proteins represent important steps in understanding structure and function relationship of membrane proteins (Kalipatnapu and Chattopadhyay, 2005).

To carry out the solubilization of the protein, three different detergents were used; n-Dodecyl beta-D-maltoside (DDM), Decyl β-D-maltopyranoside (DM) and Triton X-100.

The soluble fraction of each sample treated with the different detergents was loaded into the gel, and the fraction of the pellet resuspended (figure 39). The obtained results showed a better performance with the use of the DDM detergent, since it shows better

solubility than the rest of detergents (lane 8) and the amount of pellet obtained is lower (lane 9).

Unfortunately, there was not enough protein to carry out the purification of the glucose transporter GlcH, so the conditions were further optimized to obtain a higher level of expression of the target protein.

Optimization of GlcH expression

In order to achieve a higher level of expression, different culture media, growth temperatures and IPTG concentrations were used, as well as different induction times, always using the pcAM8 plasmid, which was the only one that showed a good level of expression. When growth temperatures of 30 °C and 37 °C were used, the protein showed a strong degree of denaturation and degradation.

The best result was obtained when using LB lac medium. This culture medium does not need the addition of IPTG, since it contains lactose, which induces the plasmid expression. Several temperatures and times were tested until it was found that the best conditions were to carry out the expression of the protein at 20 ºC with an induction time of 21 hours (figure 40).

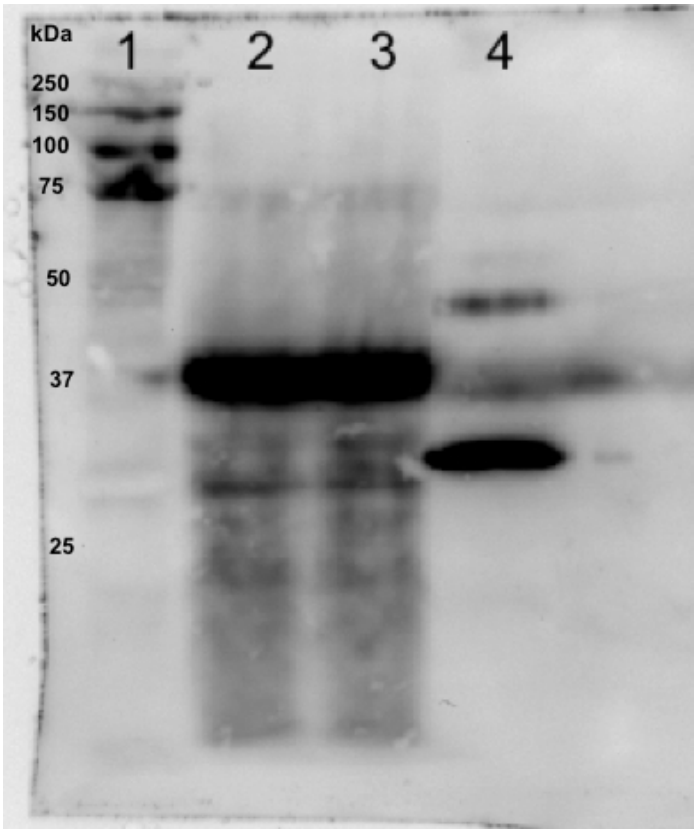


Figure 40. Optimization of GlcH expression. *E. coli* Rosetta expressing the pcAM8 plasmid. Lane 1, size marker. Lanes 2 and 3, cytosolic fraction of cell extracts from *E. coli* Rosetta cultures overexpressing the GlcH protein. Lane 4, a positive control 1 µg of a 30 kDa protein was used.

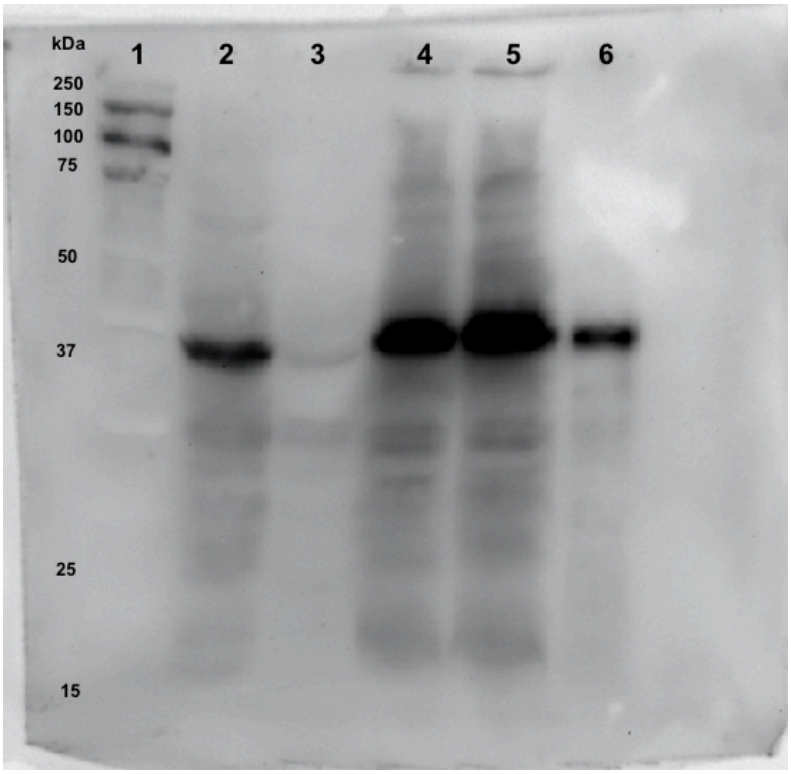


Figure 41. Optimization of GlcH expression. *E. coli* Rosetta expressing the pcAM8 plasmid. Lane 1, size markers. Lane 2, cytosolic fraction of cell extracts from *E. coli* Rosetta cultures overexpressing the GlcH protein. Lane 3, supernatant (ultracentrifugation). Lanes 4 and 5, membrane fraction. Lane 6, a positive control of a 37 kDa protein was used.

According to the obtained results, the membrane fraction shows a good grade of expression, this fraction will be used in order to carry out the purification of this membrane transporter

Purification of overexpressed GlcH from *Prochlorococcus* sp. SS120

In order to purify the GlcH transporter, an overnight culture (500 mL) was used to inoculated 10 liters of Lb Lac, the optical density was adjusted at 600 nm 0.1 OD, as described in the previous section and in the Materials and Methods 6.5 section.

To carry out the purification, the Econo-Column® Chromatography Column (*Bio-Rad)*, 1.5 × 20 was used together with 10 mL of Ni-NTA resin (*Thermo-Scientific*) as described in Materials and Methods. The obtained fractions were subjected to Western blot to identify the presence of GlcH. The results are shown in figure 42.

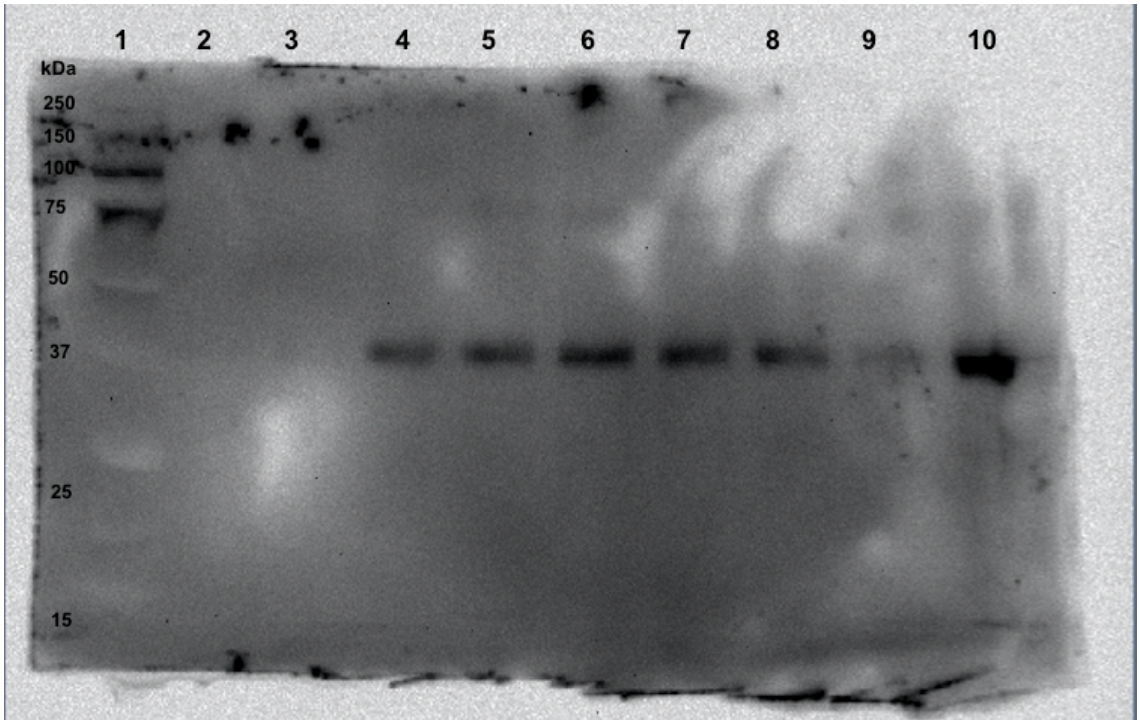


Figure 42. Western blot of the different fractions obtained after chromatography of the cell extracts on Ni-NTA. Lane 1, size markers. Lanes 2 to 9 correspond to different fractions obtained in the purification. Lanes 4 to 8 showed a good level of expression. As a positive control of a 37 kDa protein was used (lane 10).

Figure 42 shows that GlcH was produced at a high concentration and with no signs of degradation. These results obtained are very promising, and in a later stage I will continue the work in order to determine the structure of the protein by crystallographic techniques, in collaboration with Dr. Archer and her team.

Effects of glucose addition on the proteome of *Synechococcus* and *Prochlorococcus* strains

The objective of this work was to study the effect of glucose availability on the proteomic profiles of several model cyanobacterial strains of the genera *Synechococcus* and *Prochlorococcus*, with the goal of gaining a further understanding of the proteomic effects of glucose addition on those strains. We used the same experimental approach as in the metabolomic studies, by performing experiments of glucose addition (100 nM and 5 mM) to *Prochlorococcus* strains PCC 9511, SS120, MIT9313, and *Synechococcus* strains WH7803, WH8102 and BL107. However, we could only analyze the results from two *Synechococcus* and one *Prochlorococcus* strains, due to technical problems with the proteomics equipment. The remaining experiments will be analyzed and used to prepare a manuscript showing an integrated analysis of the results for all six analyzed strains.

Synechococcus sp. WH8102

A label free quantitative proteomics approach allowed the quantification of 1108 protein groups identified using at least 2 unique peptides. The analysis revealed 619 protein groups that were significantly up- or down- regulated, using the criteria of a minimum two-fold change, $q < 0.05$ and p value < 0.05.

The principal components analysis groups the samples based on light availability, obtaining two distinct groups (figure 43).

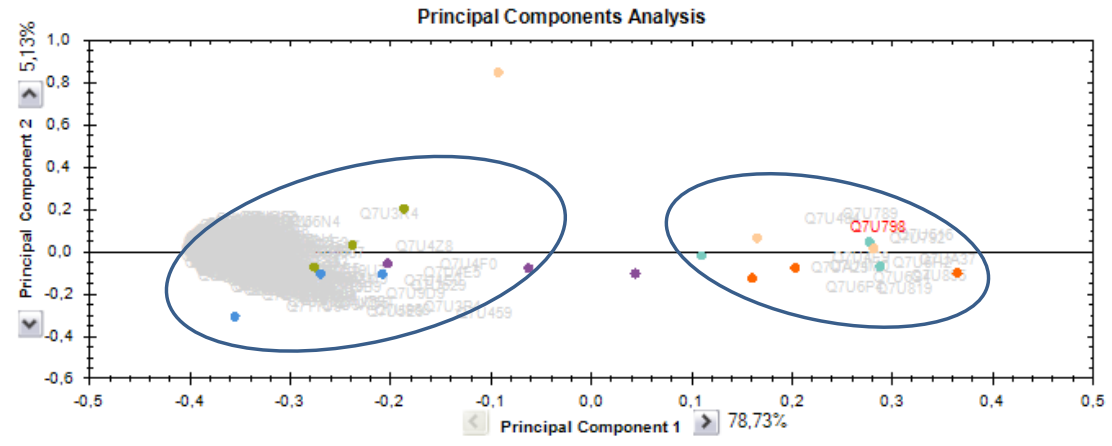


Figure 43. Principal components analysis of the *Synechococcus* sp. WH8102 proteomics dataset. Samples are separated according to the availability of light. The color used in dots corresponds to the different conditions of the study, as shown in the table above.

The results show that the relative abundance of proteins related with photosynthetic apparatus: photosystem II (Q7U480), photosynthetic pigments, C-phycoerythrin (Q7U4Q0) and R-phycocyanin II (Q7U4P4), decreased after 5 mM glucose addition and under darkness (independently of glucose concentration) (figure 44).

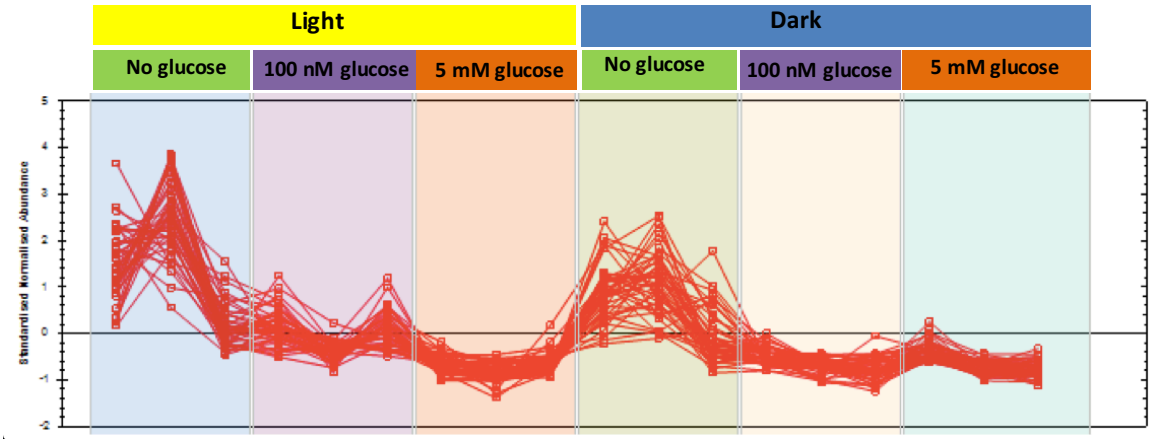


Figure 44. Protein abundance pattern shared for some proteins related to photosynthesis and ATPase.

Figure 44 shows a cluster grouping some proteins related to photosystems, some photosynthetic pigments and ATPase with the same response to darkness and glucose availability. It can be appreciated that those proteins decrease under darkness and even more in the presence of glucose either in the light or in darkness.

In connection with the metabolic map of *Synechococcus* sp. WH8102, it has been found that many metabolic pathways vary, namely the TCA cycle, pentose phosphate metabolism, amino sugar and nucleotide sugar metabolism, glycolysis, pyruvate metabolism, nitrogen metabolism, carotenoid biosynthesis.

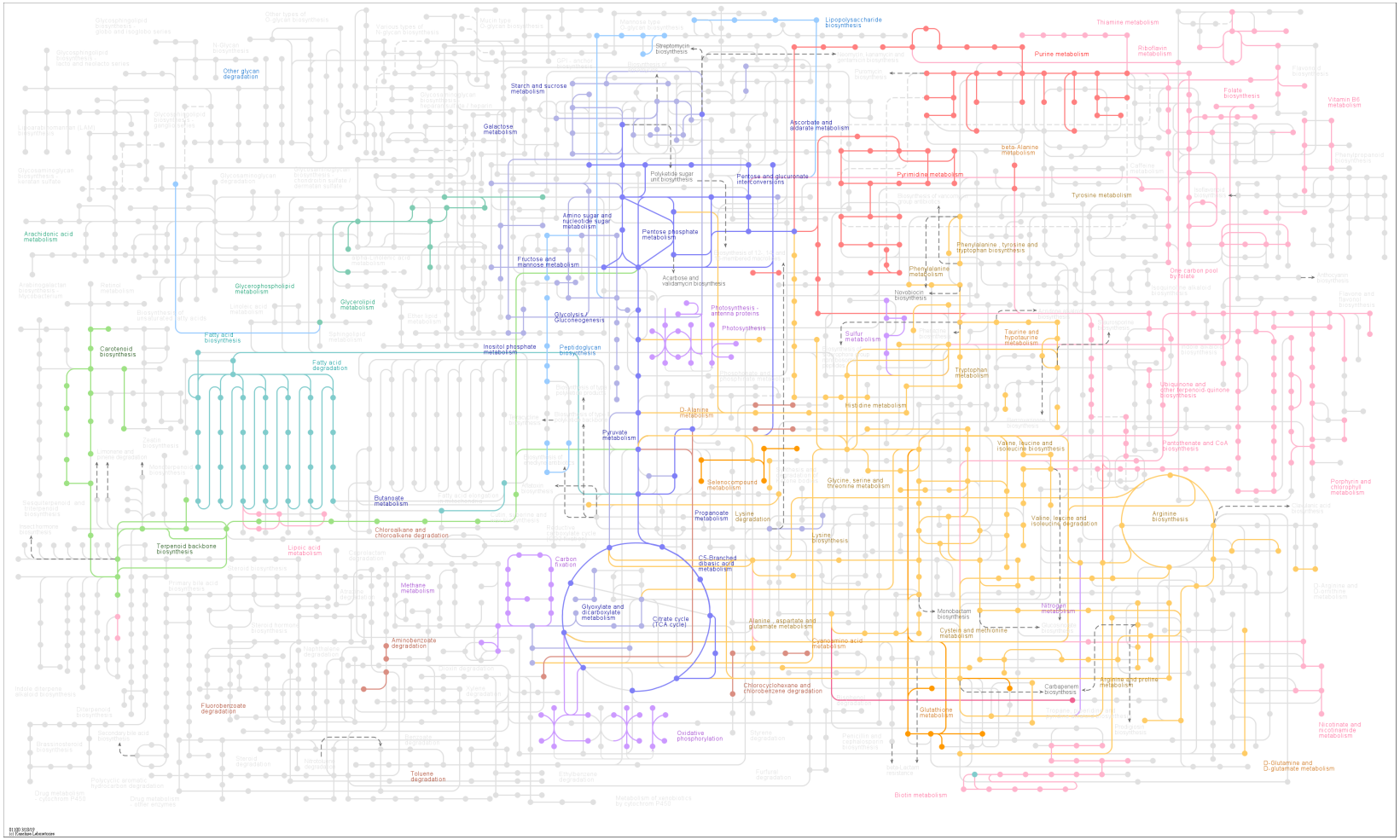


Figure 45. Metabolic map of *Synechococcus* sp. strain WH8102. The map highlights metabolic pathways that have undergone changes after glucose addition.

***Synechococcus* sp. BL107**

Label free approach allowed the quantification of 312 protein groups identified using at least 2 unique peptides. The analysis revealed 140 protein groups that were significantly up- or down- regulated, using the criteria of a minimum two-fold change, q<0.05 and p value < 0.05.

Principal components analysis groups the samples based on glucose availability.

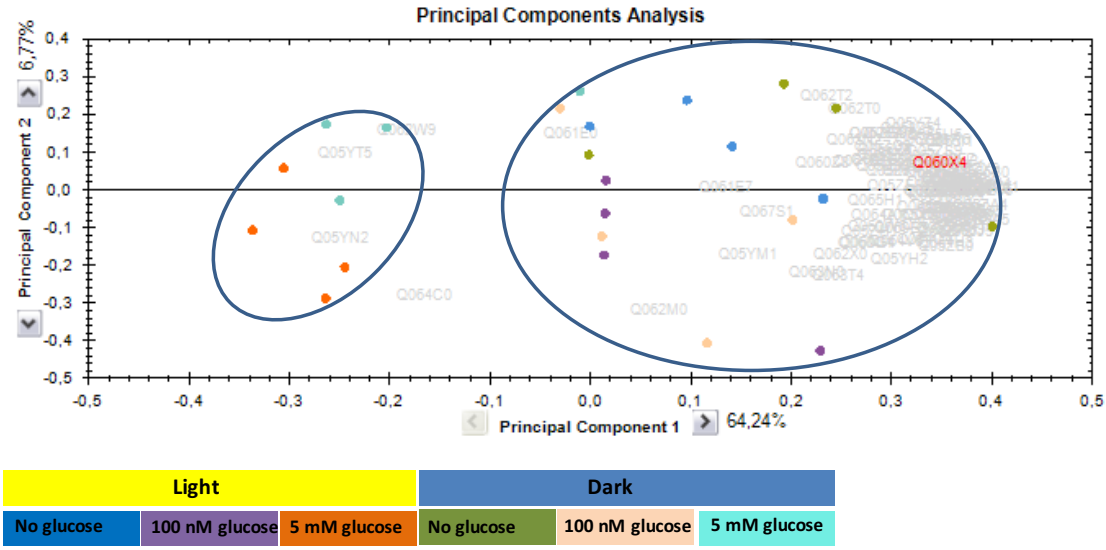


Figure 46. Principal components analysis of the *Synechococcus* sp. BL107 proteomic dataset. Samples are separated according to glucose availability. The color used in dots corresponds to the different conditions of the study, as shown in the table above.

Figure 47 shows that the relative abundance of proteins related with photosynthetic apparatus (Q066J5, Q061B7), photosynthetic pigments (C-phycoerythrin Q05ZA5), (phycocyanin Q05ZB0) and ribosomes (Q061P8, Q061P9) decreased under darkness and in the presence of glucose, the decrease being more pronounced after 5 mM glucose addition, regardless of light or darkness.

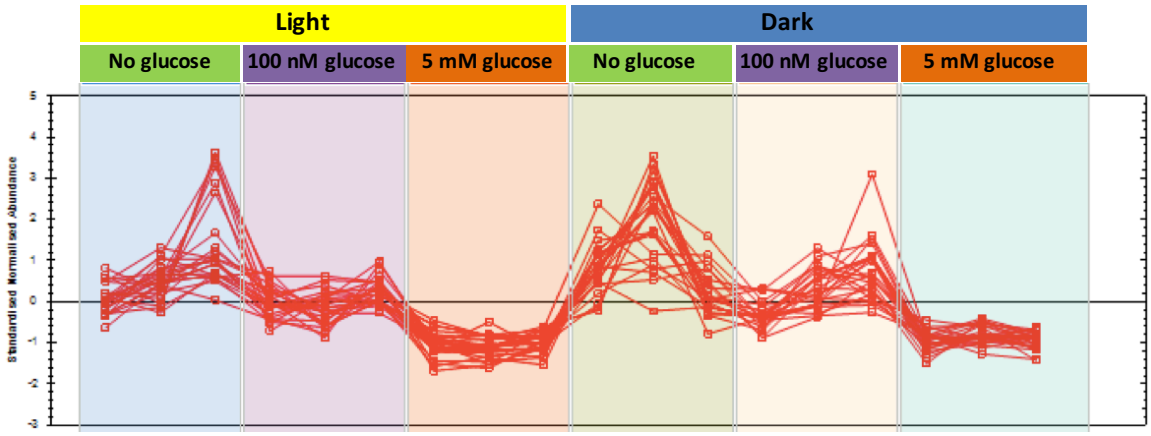


Figure 47. Protein abundance pattern shared for some proteins related to photosynthesis and ATPase.

The results set out in figure 47 show a cluster grouping some proteins related to photosystems, some photosynthetic pigments and ATPase that show the same response to the studied conditions, that is, darkness and glucose availability. It can be appreciated that those proteins decreased under darkness with glucose, and even more in the presence of glucose either in the light or in darkness.

***Prochlorococcus* sp. SS120**

Label free quantification allowed the quantification of 1031 protein groups identified using at least 2 unique peptides. The analysis revealed 538 protein groups that were significantly up- or down- regulated, using the criteria of a minimum two-fold change, $q<0.05$ and p value < 0.05.

The data referred to main components analysis groups in *Prochlorococcus* are not shown because they were obtained from one biological replica (due to the technical issues with the proteomics equipment mentioned above), although according to the data obtained, the distribution of the samples is made based on glucose availability.

Results show the relative abundance of proteins related with photosystem II (Q7VCG9, Q7VD76), photosynthetic pigments (C-phycoerythrin Q7VDN2), divinyl chlorophyll a/b (Q9L8M5) and ribosomes (Q7VA06, Q7VAY3). All these proteins decreased under darkness and in the presence of 5 mM glucose.

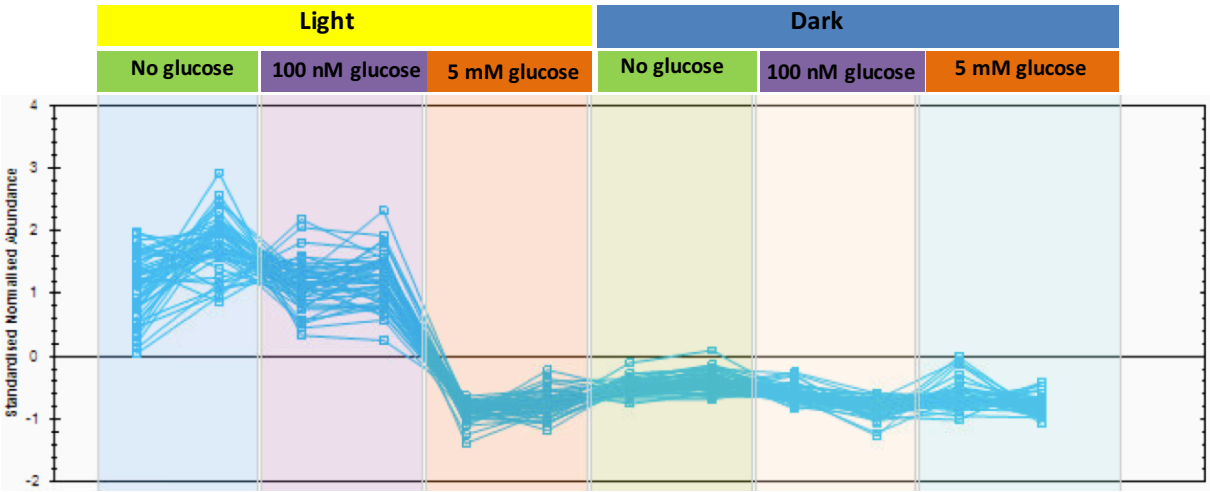


Figure 48. Protein abundance pattern shared for some proteins related to photosynthesis and ATPase.

Figure 48 shows a cluster grouping some proteins related to photosystems, some photosynthetic pigments and ATPase that show the same response to the studied conditions, that is, darkness and glucose availability. It can be appreciated that those

proteins decrease under darkness and even more in the presence of glucose either in the light or in darkness.

In connection with the metabolic map of *Prochlorococcus* sp. SS120, it has been found that many metabolic pathways vary, namely the TCA cycle, pentose phosphate metabolism, amino sugar and nucleotide sugar metabolism, glycolysis, pyruvate metabolism, nitrogen metabolism, carotenoid biosynthesis.

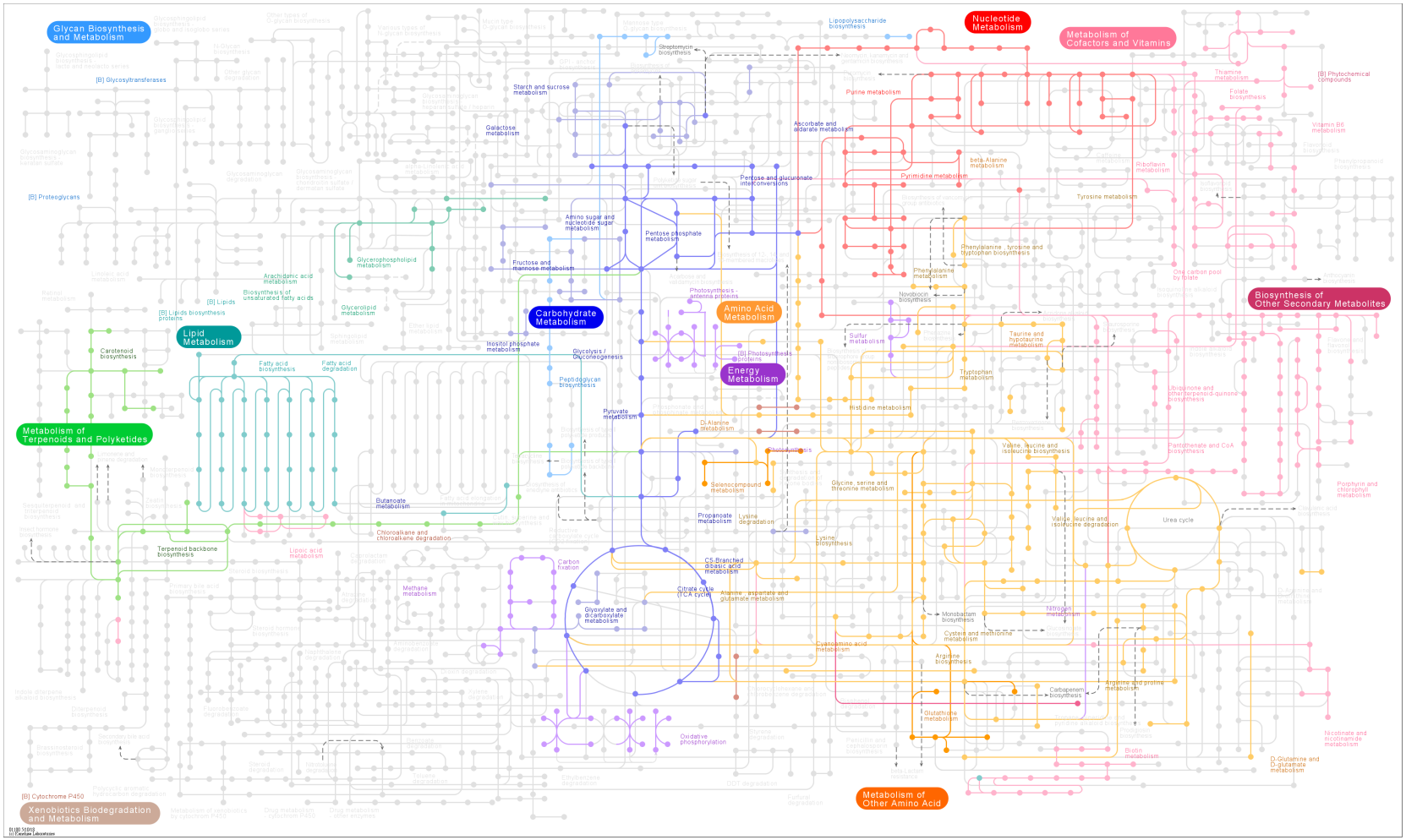


Figure 49. Metabolic map of *Prochlorococcus* sp. SS120. The map highlights metabolic pathways that have undergone changes after glucose addition.

Effects of glucose addition on the metabolome of *Prochlorococcus* and *Synechococcus* strains

The objective of the study was to understand the effect of glucose availability on the metabolomic profiles of various cyanobacterial strains of the genera *Prochlorococcus* and *Synechococcus*, with the goal of gaining a further understanding of the metabolic effects of glucose addition on those strains. Representative strains of both genera were selected, in order to allow a comparative analysis of the glucose effects in strains adapted to different environmental conditions.

The study consisted of 18 triplicate samples, representing six cyanobacterial strains: 3 *Prochlorococcus* (PCC 9511, SS120 and MIT9313) and 3 *Synechococcus* (WH7803, WH8102 and BL107) under three conditions, including control with no additions (C), 100 nM glucose (100), and 5 mM glucose (5). For each strain, the two glucose conditions were statistically compared with respect to the respective control condition. Cultures were grown to reach the exponential phase, as indicated in Materials and Methods, then divided in three identical aliquots of 3000 mL. Glucose at the indicated concentrations was added at 0 time to one of those aliquots, keeping the other one without addition as control.

The 54 cyanobacterial cell samples obtained by following this strategy were frozen in liquid nitrogen and stored at -80º C. Once the samples for all strains were available, they were sent to the European headquarters of Metabolon (Potsdam, Germany), which performed the metabolome determinations and the quantitative analysis of data.

Table 36. Cyanobacterial strains and conditions used in this study.
(N = 3 for all groups)

SPECIES	STRAIN	CONTROL	100 nM GLUCOSE	5 mM GLUCOSE
Prochlorococcus	SS120	SS_C	SS_100	SS_5
	PCC 9511	PC_C	PC_100	PC_5
	MIT9313	MI_C	MI_100	MI_5
Synechococcus	BL107	BL_C	BL_100	BL_5
	WH7803	WH7_C	WH7_100	WH7_5
	WH8102	WH8_C	WH8_100	WH8_5

The main changes observed in the metabolomic changes caused by the addition of glucose according to the heat map are:

Amino acid metabolism

Amino acid metabolism overall represented a strong differentiator of *Prochlorococcus* and *Synechococcus*. *Prochlorococcus* strains accumulated much higher levels of most proteinogenic amino acids in the 5 mM glucose samples, but *Synechococcus* strains only showed this effect for a limited number of amino acids. A few amino acids (tyrosine, glutamine, glutamate, cysteine) tended to be increased in *Synechococcus* strains, but most were not affected. Asparagine was the exception, in that it was not induced in any of the genotypes. One of the greatest fold-change increases under high glucose was for the strong osmolyte proline, but only in *Prochlorococcus* strains. This is in contrast to the significant induction of trehalose primarily in *Synechococcus* strains, a compound with similar osmolytic properties.

Amino acid catabolism

Catabolites of amino acids (as well as nucleotides) were generally lower in the high glucose samples, for several catabolites of tryptophan, tyrosine, arginine, and lysine, such as kynurenine, indole-3-carboxylate, o-tyrosine, 2-oxoarginine and pipecolate, this reflects the general shift into a more anabolic state.

Lipids

Several lipids were increased by glucose addition, especially with the highest glucose concentration, significantly in *Prochlorococcus* sp. SS120 and *Prochlorococcus* sp. PCC 9511. In *Synechococcus* strains, the most significant changes were found in *Synechococcus* sp. BL107 and *Synechococcus* sp. WH7803.

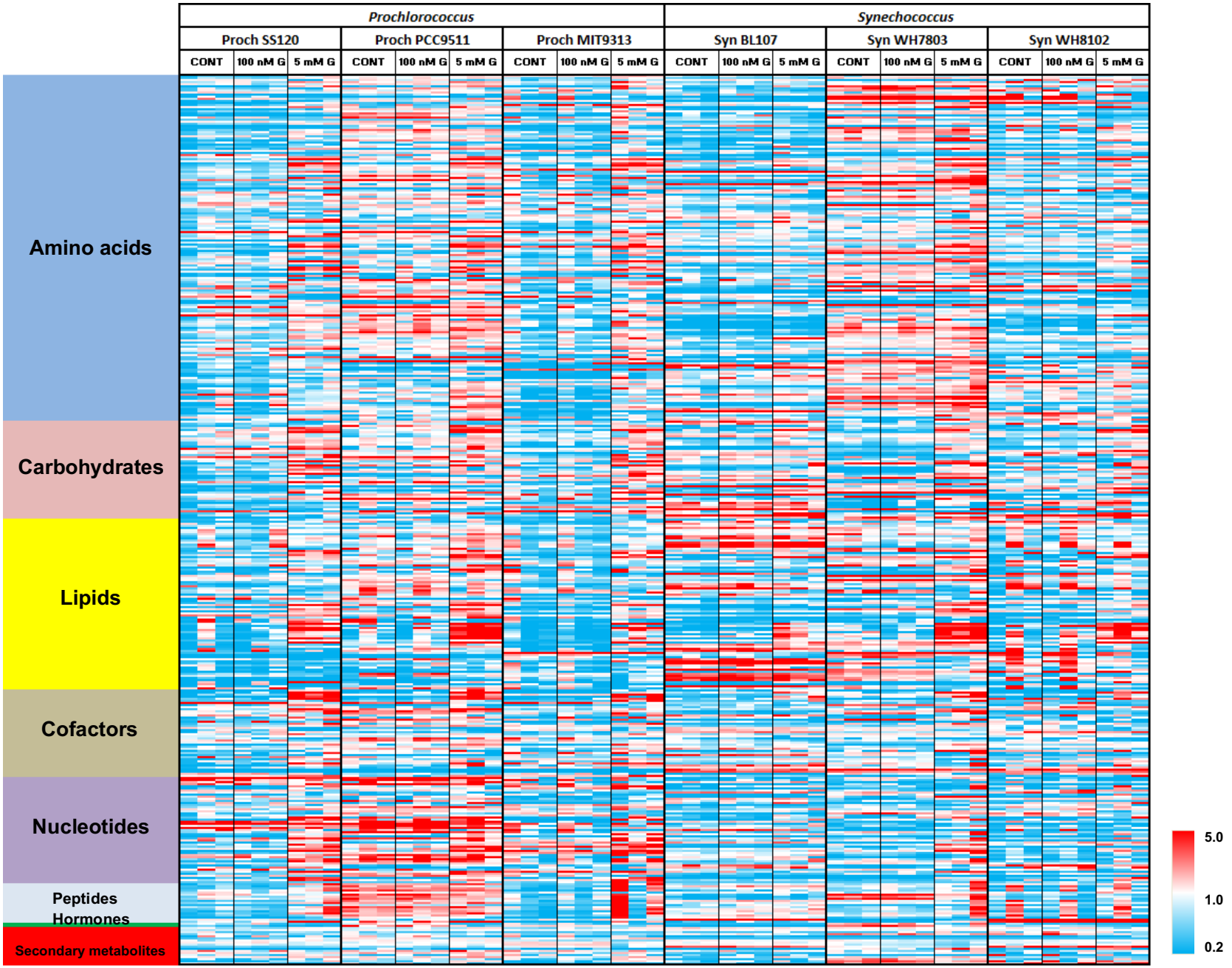
Cofactors

Many cofactors were increased by the higher glucose concentration, again consistent with a more anabolic state. This was more obvious in the *Prochlorococcus* strains and in *Synechococcus* sp. WH7803 among the *Synechococcus* strains. Among the cofactors, intermediates in the biosynthetic pathway for coenzyme A showed very dramatic induction in the high glucose samples. CoA is central to a wide range of enzymatic reactions and pathways, and servers as the primary acyl-carrier in lipid metabolism. Levels of CoA were >60-fold induced in *Prochlorococcus* sp. PCC 9511, and >10-fold induced in all the lines except for *Synechococcus* BL107, which was only induced 3.5-fold. However, final induced levels in *Prochlorococcus* sp. MIT9313 (relative to other strains) were lower than in *Synechococcus* sp. BL107.

Nucleotide metabolism

Biosynthetic precursors for both purines and pyrimidines were strongly induced by high glucose in all lines. This could suggest that the synthesis of nucleic acids in the cells increases due to the anabolism produced by the addition of glucose. The effects were more pronounced in *Prochlorococcus* strains.

Figure 50 shows a heat map analysis of the results obtained after metabolome quantitations, organized by type of metabolites.


Prochlorococcus
Synechococcus
Proch SS120
Proch PCC9511
Proch MIT9313
Syn BL107
Syn WH7803
Syn WH8102
CONT
100 nM G
5 mM G
Amino acids
Carbohydrates
Lipids
Cofactors
Nucleotides
Peptides
Hormones
Secondary metabolites
5.0
1.0
0.2

The results show that 5 mM glucose addition led to a strong metabolic shift toward overall anabolic patterns in all the studied cyanobacterial strains. We observed that 469 metabolites increased their concentration with the higher glucose concentration, and a decrease of 124 metabolites was produced, while 100 nM glucose had no measurable effect on any of the strains. The patterns of biosynthetic activity induction were stronger in all the *Prochlorococcus* strains, where most amino acids concentration were significantly increased in samples subjected to 5 mM glucose, but this pattern was much weaker, and often absent, in *Synechococcus* strains. The BL107 strain was notably the least affected by the provision of high glucose concentration, while WH7803 showed significantly higher basal pools of many metabolites, especially amino acid compounds.

In this part of the manuscript, we will focus on the study of carbohydrates, especially those that are more related to glucose metabolism in cyanobacterial strains. Specifically, we payed attention to the following metabolic pathways: glycolysis (figure 51), TCA cycle (figure 52), Calvin cycle and pentose phosphate pathway (figure 53).

In figure 51 the effects of glucose addition on the glycolysis pathway metabolites are shown. The main conclusion is that intracellular glucose pools were managed differently in *Prochlorococcus* (PC) vs. *Synechococcus* (SC) strains. It is striking that in *Prochlorococcus* samples the cellular levels of glucose were lowest in the high glucose treatment; in the *Synechococcus* strains there was no difference between the treatments, but it was clear that *Synechococcus* sp. BL107 maintained the highest levels of glucose.

Levels of dihydroxyacetone phosphate (also found in the photosynthetic pathways) showed a different effect, *i.e.* higher in *Synechococcus* samples subjected to 5 mM glucose than in *Prochlorococcus* samples. It may also be significant that the *Synechococcus* strains tended to accumulate trehalose, a disaccharide with strong osmolyte properties (Scanlan et al., 2009). Despite these strain differences in the early intermediate pools, all samples showed larger pools of phosphoenolpyruvate, pyruvate, lactate, and acetyl-CoA in samples subjected to high glucose concentrations. This effect was also visible in several intermediates of the TCA cycle (figure 52), being higher in the 5 mM glucose samples for succinyl-CoA and all detected derivative compounds in this cycle (succinate, fumarate, and malate).

The effects of glucose addition were also studied in the Calvin cycle and pentose phosphate pathways (figure 53). The results showed increases at the highest glucose concentration in 3-phosphoglycerate, except in *Prochlorococcus* sp. SS120. Dihydroxyacetone phosphate showed an increase with the highest glucose concentration in *Synechococcus* samples, however there was no significant difference in the case of *Prochlorococcus*. No significant changes were found either in sedoheptulose 7-phosphate pools after glucose addition: the trend was rather linear, or a decrease in samples subjected to 5 mM glucose. On the other hand, the results obtained in sedoheptulose show that internal glucose pools were differently managed in *Prochlorococcus* vs. *Synechococcus*. Sedoheptulose did not show clear increases at higher glucose concentration in *Synechococcus*. However, *Prochlorococcus* samples showed increased sedoheptulose concentration at a higher glucose concentration. It should be noted that *Prochlorococcus* SS120 showed a much higher sedoheptulose pool increase than the rest of the strains at 5 mM glucose-treated samples. It is remarkable that the rest of metabolites produced in the Calvin Cycle and pentose phosphate pathways did not show significant changes at 5 mM glucose in *Prochlorococcus* sp. SS120.

Glycolysis

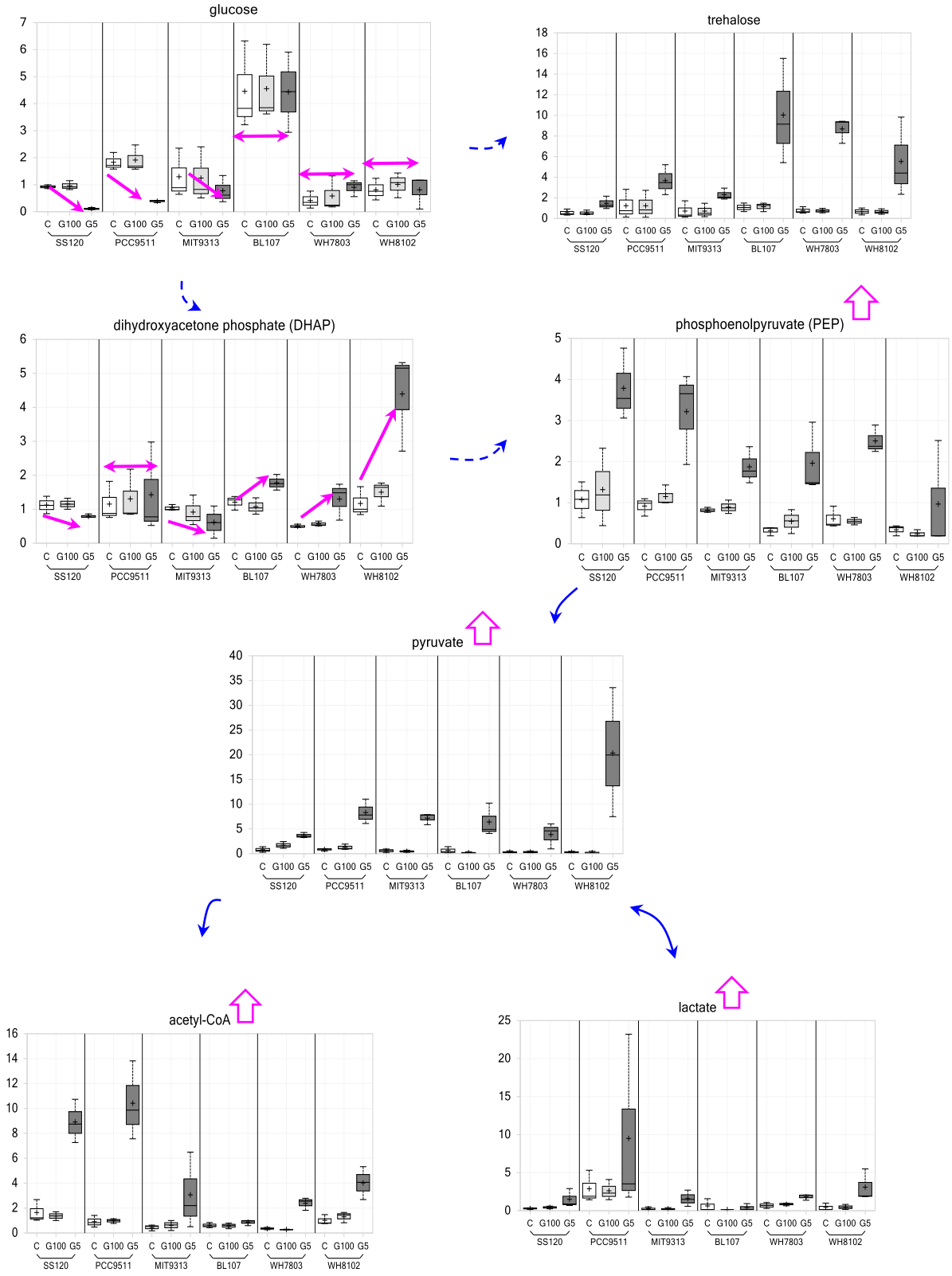


Figure 51. Effects of glucose concentration on glycolysis pathway.

TCA Cycle

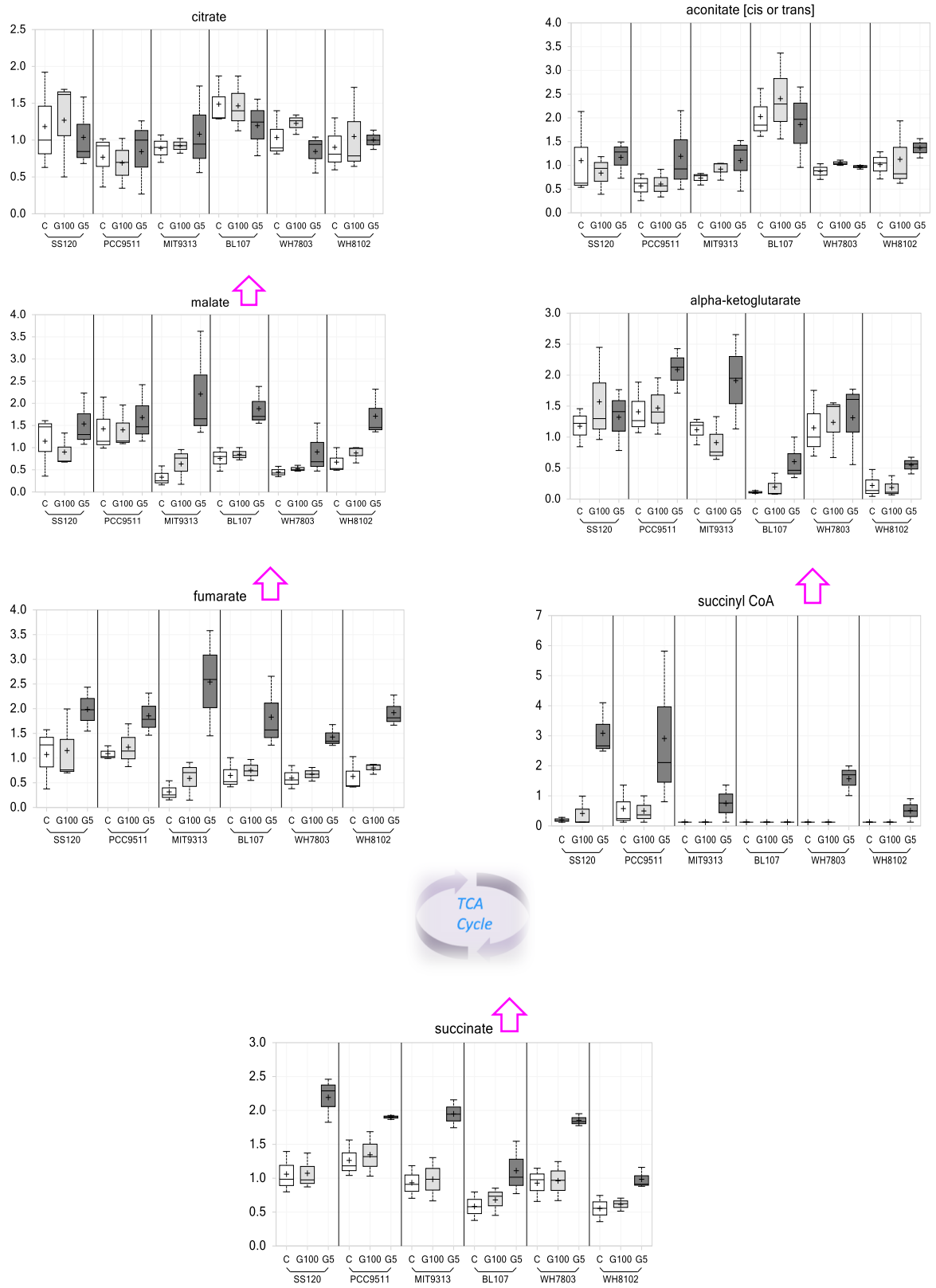


Figure 52. Effects of glucose concentration on TCA cycle pathway.

Calvin cycle and pentose phosphate

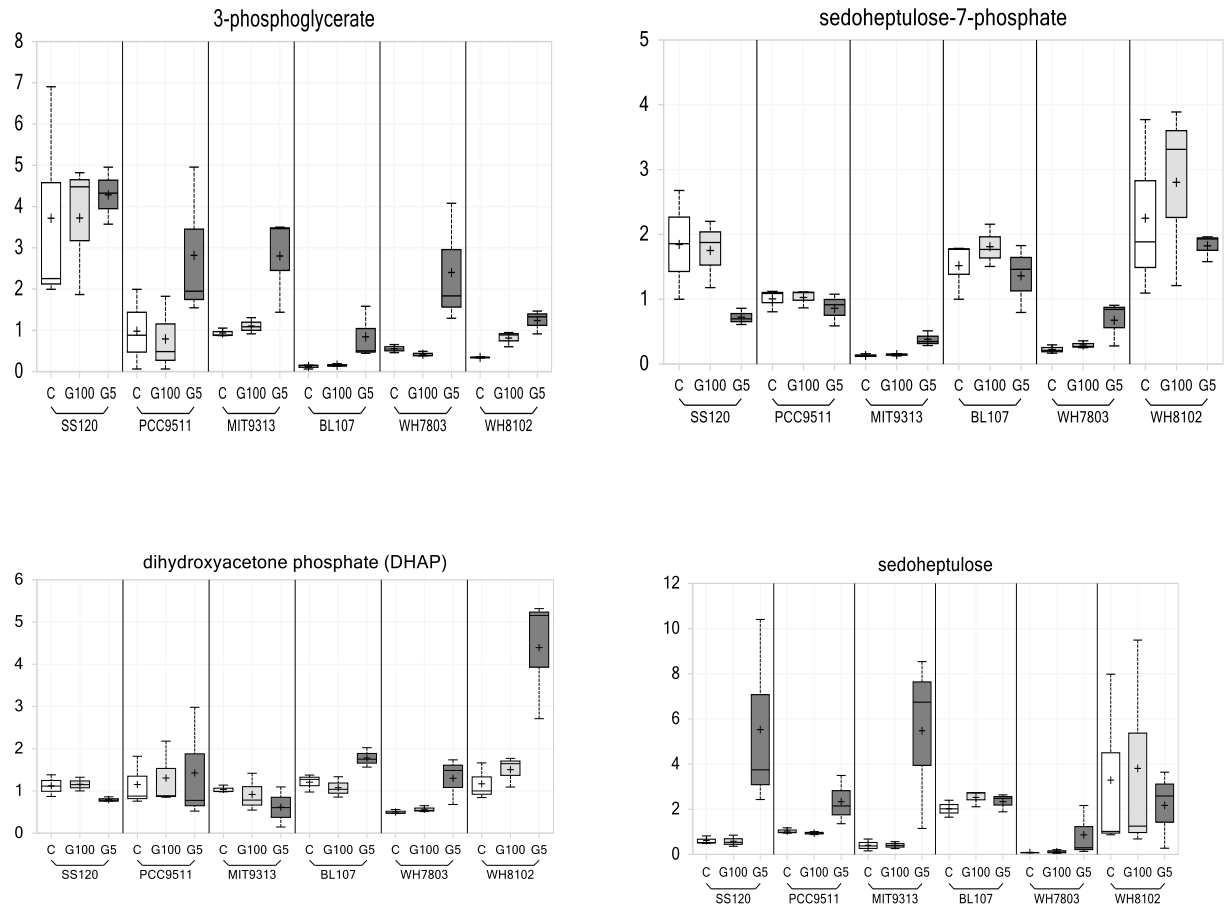


Figure 53. Effects of glucose concentration on in the Calvin cycle and pentose phosphate pathway.

DISCUSSION

Discussion

Expression of the glucose transporter gene *glcH* in *Prochlorococcus* and *Synechococcus*

The use and uptake of sugars in some types of cyanobacteria has been reported over the years. However, the molecular processes that take place to allow the uptake of glucose are little known. The first studies showed the presence of a glucose transporter due to its ability to transport fructose as well, which is toxic for some strains (as *Synechocystis* sp. PCC 6714 and PCC 6803): mutant strains resistant to fructose allowed to show the ability to take up glucose (Flores and Schmetterer, 1986). Physiological characterization of glucose uptake in *Synechocystis* showed that this process had a K_s constant in the millimolar range (Joset et al., 1988). K_s is defined as the glucose concentration at which glucose transport reaches half of its maximum value, for a given transporter. Several studies identified the gene *glcP*, encoding a sugar/H^+ symporter, as the gene encoding the glucose transporter in *Synechocystis* sp. PCC 6803 (Zhang et al., 1989). It has been shown that the gene *glcP* is homologous to other glucose transporters that have been found in animals and bacteria (Zhang et al., 1989). In *Prochlorococcus* a different type of glucose transporter was discovered (Muñoz-Marín et al., 2013), at first annotated as *melB* due to its homology with melibiose transporters described in *E. coli* (Hanatani et al., 1984), suggesting it could encode an important protein for cyanobacteria.

All these results suggest that *glcH* has been subjected to selective evolution in marine picocyanobacteria, where its physiological function has been modulated to better fit the ecological niche where they are found. From here, we hypothesized that the regulation of glucose uptake (specifically, of the expression of the gene encoding the glucose transporter) would show a certain level of diversity as well, reflecting the different habitats where model picocyanobacterial strains were isolated. This led us to research the *glcH* expression in *Prochlorococcus* and *Synechococcus* strains, subjected to changes in two key factors representative of their environmental conditions: availability of different glucose concentrations, and darkness.

The results obtained in this study confirm our hypothesis: we found differential *glcH* expression under the studied conditions (availability of different glucose

concentrations, and darkness), both in the magnitude of the observed changes and in the ways these changes happened.

According to the results obtained, we may find maximum increases of ca. 7-fold in the *glcH* gene expression in the case of *Prochlorococcus* sp. strain SS120 (low-light adapted) and *Synechococcus* sp. BL107 (figures 16 and 20), vs minimum increases of ca. 2-fold for *Prochlorococcus* TAK9803-2 and PCC 9511 (high-light adapted; figures 18 and 19), and even still lower for *Synechococcus* sp. WH7803 (figure 21).

The results are in accordance with previous studies showing higher glucose uptake levels also in low-light adapted *Prochlorococcus* strains, such as SS120 or MIT9303 (Muñoz-Marín et al., 2017). This seems to indicate that low-light adapted strains hold a higher capacity to upregulate *glcH* expression than high-light adapted ecotypes. To validate the hypothesis a larger number of strains would be required. If this happens, it would fit to the idea that glucose utilization is more useful for marine picocyanobacterial strains adapted to life with very little light available (Jiao et al., 2013), where glucose may support metabolism and allow survival under extended darkness (Coe et al., 2016).

It is noteworthy that the maximum value was found at 1,000 nM glucose in SS120, but not in BL107, where the maximum value of glucose uptake was found at 5 nM glucose. This could suggest that the regulation of *glcH* gene expression has changed, so it seems that *Synechococcus* sp. BL107 strain is more sensitive to changes in the nanomolar range of glucose, which might often happen in its habitats, that is a coastal strain which was isolated from deep samples (Six et al., 2007a; Dufresne et al., 2008), while all other *Prochlorococcus* and *Synechococcus* studied strains, show the maximum value at the highest tested glucose concentration. In any case, additional studies are necessary regarding sugar concentrations at the deep waters where *Synechococcus* sp. BL107 was isolated.

It has been shown that the transport and consumption of glucose is beneficial, in bioenergetic terms, for marine picocyanobacteria, respect to glucose synthesis *de novo* (Muñoz-Marín et al., 2013), it is profitable for them to take up glucose from the marine environment when possible. Even if they need to use energy to uptake glucose in the dark, the balance is still positive, as shown for example in the extended survival under darkness

which has been reported after glucose addition to *Prochlorococcus* cultures (Coe et al., 2016).

According to the obtained results, the levels of expression can be grouped into three patterns, some strains show a progressive increase (SS120, MIT9313, TAK9803-2), in others, there is little variation for most glucose concentrations (PCC 9511, WH7803), and finally, in the case of *Synechococcus* sp. BL107, the observed values have a peak at the mid tested concentration, unlike all other cyanobacterial strains here studied.

The results obtained confirm that *glcH* is expressed even when no glucose was added to the cultures, but its expression increased when glucose was added, suggesting that marine picocyanobacteria are monitoring the presence of glucose in the environment, so that they can upregulate glucose uptake to use it when available. The mechanisms used by *Prochlorococcus* and *Synechococcus* to detect the presence of glucose in the environment are currently unknown. Interestingly, the sensor kinase Hik31 has been shown to be involved in glucose sensing in *Synechocystis* sp. strain PCC 6803 (Kahlon et al., 2006). Otherwise, this sensor kinase is not found in all known *Prochlorococcus* and marine *Synechococcus* genomes, and hence the sensor molecule must be different in these cyanobacteria.

The level of glucose uptake also increased in *Plectonema boryanum* in the presence of glucose (Raboy and Padan, 1978). However, in other cyanobacterial strains the glucose uptake is not inducible by glucose (Beauclerk and Smith, 1978; Der-Vartanian et al., 1981). It is worth noting that the mechanisms for glucose uptake previously discovered in cyanobacteria are low affinity, and therefore it is expectable that their regulation and/or inducibility may be subjected to different rules than the very high affinity transporter encoded by *glcH* gene in marine picocyanobacteria. The large difference in the K_s values for GlcH vs GlcP transporters (about 3,000 times) (Muñoz-Marín et al., 2013) could explain why *glcH* expression is markedly upregulated at nanomolar concentrations of glucose (figures 16, 17, 18, 19, 20 and 21), in a special way in *Prochlorococcus* sp. MIT9313 and *Synechococcus* sp. BL107. At low glucose concentrations no transport has been reported in non-marine strains, it could be due that its function as a very high affinity glucose transporter has not yet been studied thus far or due to the transporter is not present

in those microorganisms. We are currently studying this topic in some model freshwater strains to make a comparative study.

The results show that darkness induced a strong decrease in *glcH* expression in all tested cyanobacterial strains (figures 22 and 23), which in some cases was almost completely arrested (i.e., *Prochlorococcus* sp. Strain MIT9313). This was in good agreement with previous results showing that glucose uptake was ca. 42% inhibited by darkness in *Prochlorococcus* sp. SS120 strain (Gómez-Baena et al., 2008). Moreover, addition of photosynthetic electron transport inhibitors also induced a significant decrease in glucose uptake: DCMU promoted a 50% decrease, whilst DBMIB abolished glucose uptake (Muñoz-Marín et al., 2017). All the above mentioned results suggest that GlcH is an active transporter (Muñoz-Marín et al., 2017), and thus marine picocyanobacterial cells are using metabolic energy in order to take up glucose, since it is beneficial in energetic terms with respect to de novo biosynthesis (Muñoz-Marín et al., 2013). Therefore, the energy limitations imposed by darkness or photosynthetic inhibitors on the metabolism of marine picocyanobacteria explain the decrease in glucose uptake and *glcH* expression.

The situation is different in freshwaters strains using the GlcP transporter, which can take up glucose in darkness. For example, *Aphanocapsa* sp. PCC 6714 was capable of glucose assimilation in darkness or under light in the presence of 10 μM DCMU. *Synechocystis* sp. PCC 6803 and *Aphanocapsa* sp. PCC 6714 were shown to grow on glucose in the presence of 10 μM DCMU (Der-Vartanian et al., 1981; Flores and Schmetterer, 1986; Schmetterer, 1990), however *Synechocystis* sp. PCC 6803 showed extremely weak growth in the light with glucose (Flores and Schmetterer, 1986). Cyanobacterium *Plectonema boryanum* showed a 20% decrease in glucose uptake after cells were subjected to darkness (Raboy and Padan, 1978). Growth on glucose, either under light or darkness, has not been reported in marine picocyanobacteria. However, it has been shown that 10 mM glucose, fructose and sucrose were tolerated and seemed to slightly prolong survival, but it had no major beneficial effect on the growth of *Prochlorococcus marinus* PCC 9511 (Rippka et al., 2000). Nonetheless, field studies in the oligotrophic South Pacific Ocean suggested that glucose could support the growth of natural populations of *Prochlorococcus* (Moisander et al., 2012).

The effect of light has been shown to stimulate the uptake of organic compound in marine picocyanobacteria, including amino acids and ATP (Michelou et al., 2007; Mary et al., 2008b; Duhamel et al., 2012; Gómez-Pereira et al., 2013). In addition, a study has been reported with natural picocyanobacterial populations in the western tropical South Pacific Ocean, where it has been observed that light enhanced cell specific glucose uptake by ca. 50% for *Prochlorococcus* and *Synechococcus* (Duhamel et al., 2018). These results agree with ours, Duhamel and collaborators proposed that variability in light availability (derived from factors such as diel sunlight rhythms, cloud average…) may significantly impact glucose uptake in marine cyanobacteria. Given the great similarity of their observations to the stimulation of *glcH* gene expression by light in *Prochlorococcus* and *Synechococcus* (figures 22 and 23), we propose that light availability controls glucose uptake in marine picocyanobacteria by transcriptional regulation of *glcH*.

Moreover, studies using photosynthetic transport inhibitors suggest that incorporation of glucose into the cells can be partly supported by ATP produced by the cyclic electron transport around photosystem I. This would allow the upkeep of ca. 50% glucose uptake when photosystem II is not working, providing an important level of metabolic flexibility in marine picocyanobacteria (Muñoz-Marín et al., 2017; Duhamel et al., 2018).

In the different cyanobacteria studied here, we found that darkness had a stronger effect on *glcH* expression in *Prochlorococcus* than in *Synechococcus* (figures 22 and 23). If this situation is confirmed with subsequent studies, using a greater number of strains, it could help to understand why *Synechococcus* seems to have a longer dark-survival capacity than *Prochlorococcus* (Coe et al., 2016). The effect of darkness would allow a higher glucose uptake level in *Synechococcus* than in *Prochlorococcus*, providing carbon and energy when photosynthesis is not possible, and therefore adjudicating and advantage to *Synechococcus* over *Prochlorococcus* when subjected to environmental conditions of very low or not light at all.

Altogether, the results that have been shown in this work suggest that *Prochlorococcus* and *Synechococcus* have modified the regulation of *glcH* transcription in their evolution, what agrees with a recent metapangenomic publication, which showed a small set of core genes occurring in hypervariable genomic islands of *Prochlorococcus*

populations, all of them related to sugar metabolism (Delmont and Eren, 2018). This study suggested that a high sequence diversity of sugar metabolism genes could confer benefits to *Prochlorococcus*. Therefore, such a high sequence diversity could be linked to the diversity observed in the transcriptional control mechanisms. However, further studies and additional work are needed to understand the physiological relevance of sugar uptake and utilization for marine picocyanobacteria.

Directed mutagenesis of *glcH* from *Prochlorococcus* marinus SS120

The improved overlapping-extension PCR based method used in this study is generic and can be employed to create various kinds of in vitro mutations, including point mutations, nucleotide substitutions, insertions or deletions. Moreover, this method is cheap, easy to use and accurate (Kanoksilapatham et al., 2007). It has been widely used to produce amino acid mutations in a multitude of cyanobacteria transporters. Overlapping-extension PCR was used to study the involvement of a CbbR homolog in low CO_2-induced activation of the bicarbonate transporter operon in *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7942 (Omata et al., 2001). *Anabaena* sp. PCC 7120 is another cyanobacterium where overlapping-extension PCR has been highly developed. Site-directed mutagenesis experiments demonstrated that the ManR specifically binds to the promoter of a Nramp transporter gene in *Anabaena* sp. PCC 7120 (Huang and Wu, 2004). Moreover, the gene *devA* of the filamentous heterocyst-forming cyanobacterium *Anabaena sp. strain* PCC 7120 encodes a protein with high similarity to ATP-binding cassettes of ABC transporters (Fiedler et al., 1998). The additional genes, denomined *devB* and *devC*, encode proteins with similarities to membrane fusion proteins (DevB) of several ABC exporters and to membrane-spanning proteins (DevC) of ABC transporters. Site-directed mutation was used in each of the three genes and resulted in identical phenotypes (Fiedler et al., 1998).

From the study of the MelB transporter of *Salmonella typhimurium* (Ethayathulla et al., 2014), and subsequent alignment with other sequences of cyanobacteria, two residues were selected to carry out the directed mutagenesis. These residues are involved in sodium binding to the transporter, and formation of "ionic locks". We report herein the directed mutagenesis of the essential residues Asp52 and Arg134 of the GlcH transporter,

which are expected to affect the glucose transport system. Our initial studies of glucose transport by using these mutants provided unclear results, which might be due to the instability in the expression of GlcH from *Prochlorococcus marinus* strain SS120 in *Synechococcus elongatus* PCC 7942. This fact had already been observed in the process of construction of the *S. elongatus* strains which allowed to demonstrate that the Pro1404/*glcH* gene encodes a high affinity glucose transporter in *Prochlorococcus marinus* SS120 (Muñoz-Marín et al, 2013). Further studies will be carried out in the coming months in order to clear out the impact of the mutations generated in this work on the glucose uptake process.

Overexpression of GlcH protein

It has been reported that up to 30% of the proteins encoded in prokaryotic and eukaryotic genomes are membrane proteins (Wallin and von Heijne, 1998). The study of membrane proteins is considered a great challenge and presents an enormous difficulty due to its hydrophobicity and low natural abundance, which contributes to the relatively low number of membrane protein structures deposited in the protein databank (www.rcsb.org) (Ma et al., 2013). Generally, functional and structural studies require milligram quantities of pure protein, and optimization of conditions for the overproduction and purification of membrane proteins is tedious. In recent years, efforts have been made to develop efficient methods and high performance for the production of membrane proteins, however there are numerous obstacles to carry them out (Bill et al., 2011; Mancia and Love, 2011; Kang et al., 2013).

First, membrane proteins, due to the low natural abundance they present, require heterologous overexpression of their genes that is generally accompanied by toxicity to the host cell. Secondly, the choice of suitable detergents for the extraction of proteins from the cell membrane is complicated. Finally, for X-ray crystallographic studies, the protein sample must be pure and homogeneous, since heterogeneity can hinder the formation of well-ordered crystals (Ma et al., 2013)

The production of integral membrane proteins is a complicated process, and often constitutes an obstacle for further studies. Previous studies of overexpression in *E. coli* focused mainly on cell strains, temperature and inductor concentration (Ma et al., 2013) Other studies explored several host systems and protein fusion tags for the production of membrane proteins (Tate et al., 2003; Drew et al., 2006; Hammon et al., 2009; Hsieh et al., 2010; Bernaudat et al., 2011; Sonoda et al., 2011; Hsu et al., 2013).

When we expressed *glcH* in the plasmid pcJA6A, the size we obtained for the expressed GlcH protein when it was checked by Western blot did not correspond to the expected size. The protein sizes observed in the Western blot were ca. 20 kDa (figure 38).

However, the plasmid pcAM8 showed a good level of expression. The expected size of the expressed protein was 50 kDa, and that observed in the Western blot was 37 kDa (figure 39). This value is valid, since migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that does not correlate with molecular weights, called ‘‘gel shifting’’, appears to be common for membrane proteins. It has been shown that the helix-loop-helix “hairpins” migrate at rates of 10 to 30% slower than proteins with their actual formula weights on SDS-PAGE prepared with 3.4–10% SDS (Rath et al., 2009). Therefore, the plasmid pcAM8 provided a correct way to carry out large scale studies in order to overexpress the Pro1404 glucose transporter.

The detergents, being amphipathic molecules, are soluble in aqueous solution and can be inserted in hydrophobic phases, including the membranes. The critical micelle concentration reflects the solubility of the individual detergent monomer in water (Lenoir G. et al., 2018). Membrane proteins have the tendency to form aggregates, even in the presence of detergents, resulting in the reduction of efficiency of subsequent purification techniques (Von Jagow G. et al., 1994). Therefore, it is important to choose the right detergent and adjust the amount of it to avoid problems.

An essential criterion for the effective purification of membrane proteins is that the protein must be removed from the native membrane and dispersed in a buffered detergent solution using amphipathic detergents. (Jones et al., 1987; Seddon et al., 2004; Kalipatnapu and Chattopadhyay, 2005). To carry out the solubilization of the GlcH

protein, 3 types of detergents were used; DDM, DM and Triton-X. They all belong to the family of non-ionic detergents, characterized by not having charge in the polar part and they are the most frequent to solubilize membrane proteins (Lee et al., 2018). Of the 3 detergents, DM and DDM showed greater solubilization capacity of the protein, but we decided to opt for DDM to carry out the purification in large-scale studies, because it has been characterized as a mild detergent suitable for crystallization (Kim and Ng, 2017). The length of the aliphatic chain can also vary, although detergents commonly used in membrane protein stabilization and solubilization usually have aliphatic chains of 6 to 12 carbons (le Maire et al., 2000; Lenoir G. et al., 2018).

When the solubilization process has been carried out, the protein membranes are more susceptible to degradation processes due to the action of the proteases. Therefore, the protease inhibitors phenylmethyl sulfonyl fluoride (PMSF) was used in our experiments (Smith, 2017). Similar results have recently been reported, where solubilization and extraction of membrane proteins is carried out using the DDM detergent, this detergent is considered the gold standard due to it combines good solubilization properties with mildness toward a lot of membrane proteins. Thus, DDM has been successfully employed for a large variety of membrane proteins (Guillet P. et al., 2019). Moreover, representatives β-barrel and α-helical membrane proteins were successfully stabilized with DDM detergent (Dimitri et al., 2019).

Proteomics

Proteomic analysis is a technique of interest that helps to better understand the physiological response of cells to changes in their environment. Unfortunately, there are few scientific articles related to proteomic studies in cyanobacteria (Ran et al., 2007; Teikari et al., 2015; Xiong et al., 2016), and there are even scarcer studies in marine cyanobacteria (Saito et al., 2014; Christie-Oleza and Armengaud, 2015; Christie-Oleza et al., 2015a; Mackey et al., 2015; Mackey et al., 2017; Battchikova et al., 2018; Kaur et al., 2018).

Our work is one of the first proteomics studies where the response to key environmental factors in marine environments, such as light and glucose availability, is analyzed. In addition, this study serves to complement other experiments, where we have

studied under the same conditions changes in the metabolome and in the expression of genes related to carbon metabolism (Gómez-Baena et al, 2008), including *glcH*, encoding for a high affinity glucose transporter.

Among the studies on proteomics of marine cyanobacteria, it is worth highlighting the one carried out by Oleza and coworkers reporting that marine *Synechococcus* strains have the ability to strongly modify their exoproteome, increasing the repertoire of interaction proteins when grown together with heterotroph, or decreasing exposure to prey when grown in the dark (Christie-Oleza et al., 2015b). Our studies used a different approach, focused on the response of *Prochlorococcus* and *Synechococcus* strains to the availability of glucose, under light or darkness, thus assessing two important environmental conditions for marine picocyanobacteria.

Our results confirm that *Prochlorococcus* and *Synechococcus* respond to glucose availability, showing changes that help to understand carbon metabolism in these picocyanobacteria. Together with previous results on these organisms, we provide evidences to demonstrate that glucose is not only taken up by these microorganisms, but that it is actually assimilated, inducing significant changes in the proteome and metabolome of the studied strains.

The addition of 100 nM of glucose did not promote strong changes in the proteome of *Prochlorococcus* and *Synechococcus*, this is in agreement with the report of Muñoz-Marín and coworkers (Muñoz-Marín et al., 2017) that found similar results in *Prochlorococcus* sp. strain SS120. The obtained profiles show similarity between *Synechococcus* strains, when adding a concentration of glucose of 5 mM, and in dark conditions (regardless of the concentration of glucose), the proteins related to the photosystems, photosynthetic pigments, ATPase, and ribosomes showed a strong decrease. The results obtained in *Prochlorococcus* sp. strain SS120 are similar to those found in marine *Synechococcus*, although the decrease of proteins in darkness is much more pronounced.

The addition of 5 mM glucose caused significant changes, such as a sharp decrease in some proteins related to the photosynthetic apparatus (Q066J5), photosynthetic pigments (Q7VDN2), and ribosomes (Q061P8). This is in agreement with the report of

García-Fernández and coworkers, where the addition of glucose to *Prochlorococcus* sp. strains SS120 and MED4 subjected to continuous darkness provoked a decrease in *psbA* transcript levels (the gene encoding the D1 protein of photosystem II) (García-Fernández et al., 1998). This indicates that marine picocyanobacteria change their metabolism, removing the photosynthetic machinery possibly the avoid spending resources in proteins which are not useful under darkness, or when a convenient source of carbon and energy (as glucose) is available.

We observed clear effects of darkness alone on the proteome of the analyzed strains. This is in good agreement with the metabolism of organisms which are primarily autotrophic. A high level of responsiveness to the light conditions was also reported in a study addressing the effect of different light irradiances in several *Synechococcus* strains: more than 50% of abundant proteins showed changes, varying more than two fold between the lowest and the highest irradiance. All studied strains, among them one of our studied strains (*Synechococcus* sp. strain WH8102), downregulated phycobilisome proteins when irradiance increases (Mackey et al., 2017). Our results also showed a general downregulation of photosynthesis-related proteins in *Prochlorococcus* and *Synechococcus* samples subjected to darkness.

Future work will address the changes in the proteome of the cyanobacterial strains which could not be analyzed in this manuscript, in order to obtain a more complete picture of the glucose addition effects on the proteome of *Prochlorococcus* and *Synechococcus*.

Finally, it is worth noting that there was generally a good level of correspondence between the results we obtained in the proteomic and metabolomic experiments assessing the effects of glucose availability, indicating there is a concerted response in the physiology of marine *Synechococcus* and *Prochlorococcus* in order to adapt to specific concentrations of glucose: while 100 nM glucose provoked little effects in both the proteome and metabolome (consistent with previous results reported by Muñoz-Marín and coworkers, Muñoz-Marín et al 2017), 5 mM glucose induced important changes which were consistent with the utilization of this sugar by marine picocyanobacteria.

We decided to study the effects of 100 nM glucose since it is a concentration relatively similar to that observed in oligotrophic oceans (Muñoz-Marín et al, 2013). Nevertheless, the proteomic and metabolomic response of 5 mM glucose provided insights in the actual utilization of glucose when available at high concentrations, and

allowed us to assess the physiological effects of this sugar in *Prochlorococcus* and *Synechococcus*. Other studies analyzing the effects of millimolar concentrations of glucose have shown interesting effects on the photosynthetic apparatus (García-Fernández et al, 2018) and also on the survival under darkness (Coe et al, 2016) of *Prochlorococcus*. While it is highly improbable to find this glucose concentration in the oceans, its effects are however interesting since marine picocyanobacteria might be subjected to temporary increases of glucose concentration, which might be assimilated by following the metabolic changes in different pathways here described.

Future work should assess the relative contribution of different glucose concentrations to the carbon assimilation by marine picocyanobacteria in the oceans, in order to further understand how the genetic potential for mixotrophy (Yelton et al, 2016) is exploited by these microorganisms.

Metabolomics

Metabolomic analysis is a powerful tool to better understand the physiological response of cells to changes in their environment. However, the number of metabolomics studies in marine picocyanobacteria is very small. Our omic dataset is one of the first metabolomic analysis obtained in marine cyanobacterial strains. Our results show how *Prochlorococcus* and *Synechococcus* respond to availability of glucose, providing clues for understanding the carbon metabolism in these cyanobacteria.

Intracellular pools of glycolytic intermediates, including fructose-6-phosphate, 3-phosphoglycerate, 2-phosphoglycerate, and phosphoenolpyruvate were higher under mixotrophic conditions in *Synechocystis* sp. PCC 6803 in the presence of 5 mM glucose, one of the concentrations used in our study (Yoshikawa et al., 2013). We observed similar results, where all *Prochlorococcus* and *Synechococcus* strains showed increases in phosphoenolpyruvate (PEP), pyruvate, lactate and acetyl-CoA (figure 51). However, dihydroxyacetone phosphate (DHAP) showed two different profiles; *Synechococcus* strains tended to increase DHAP, while *Prochlorococcus* strains tends to decrease or maintain the concentration of this metabolite (figure 51). As in *Synechococcus*, similar results were reported in *Synechocystis* sp. PCC 6803 after 24 h of incubation under photomixotrophic conditions (5mM glucose and bubbled with air supplemented with 1%

CO_2): DHAP increased, indicating an increase in carbon flow to the oxidative pentose phosphate pathway and glycolysis (Yoshikawa et al., 2013). In cyanobacteria, the principal route of glucose catabolism is the oxidative pentose phosphate, although the lower part of the glycolytic pathway is also involved (Pelroy et al., 1972).

Moreover, the increment in NADH content under photomixotrophic conditions also shows the enhanced flow through glycolysis. In addition, the measurement of glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities suggested that the enzymatic activities themselves were not up regulated in photomixotrophic conditions (Takahashi et al., 2008). This could indicate that the abundant supply of substrates under mixotrophic conditions drives to the enhancement of sugar catabolism.

Interestingly, internal glucose pools in *Prochlorococcus* strains tended to decrease, while *Synechococcus* strains maintained stable glucose levels. Another significant change was found in trehalose, which acts differently in *Synechococcus* and *Prochlorococcus*. *Synechococcus* tends to accumulate trehalose, whereas in *Prochlorococcus* it showed a much smaller effect (figure 51). It has been reported that *Phormidium autumnale* sp. LPP_4 and *Croroococcidiopsis* sp. accumulated trehalose in response to matric water stress. This sugar was accumulated by *Phormidium* in similar concentrations under water stress (Hershkovitz et al., 1991). It is possible that the elevated intracellular salt concentration together with the high concentration of glucose induces the production of sugars like trehalose in *Synechococcus*. In addition, results based in a computational study of the osmoregulation network in response to hyperosmotic stress of *Synechococcus* sp. WH8102 show that this organism likely uses osmolytes such as trehalose, making it more efficient and adaptable to its changing environment (Mao et al., 2010).

The carbon flow through the Calvin cycle generally decreases in *Prochlorococcus* strains after glucose addition (figure 53). This is in agreement with the report of Takahashi and coworkers (Takahashi et al., 2008) that found similar results in *Synechocystis* sp. PCC 6803. Carbon flow through the Calvin cycle seems to decrease under photomixotrophic conditions, due to the decrease in the Calvin cycle intermediates and in the photosynthetic activity. This shows that the restriction of photosynthesis is one of the strategies of *Synechocystis* sp. PCC 6803 to adapt to the mixotrophic conditions

(Takahashi et al., 2008). Our results suggest that *Prochlorococcus* could use the same strategy. The amounts of 3-phosphoglycerate, phosphoenolpyruvate and pyruvate significantly increased upon glucose addition in both *Prochlorococcus* and *Synechococcus* (figures 52 and 53), in contrast to the decrease found in *Synechocystis* sp. PCC 6803. The decrease produced in 3-phosphoglycerate, phosphoenolpyruvate and pyruvate metabolites in *Synechocystis* sp. PCC 6803 under photomixotrophic conditions, are probably caused by the repression of the activities of phosphoribulokinase and glyceraldehyde-3-phosphate dehydrogenase (Takahashi et al., 2008), so it seems that regulatory mechanisms differ depending on the type of cyanobacteria.

In addition, it would be interesting to complete this work with future studies on photosynthetic activity, since it has been reported in *Synechocystis* sp. PCC 6803 that the decrease in the amount of the Calvin cycle intermediates such as 3PGA under mixotrophic conditions was much larger that under photosynthetic ones (Takahashi et al., 2008). It could be possible that 3PGA scarcely accumulates under photomixotrophic conditions, probably by the increased metabolic flux to the oxidative pentose phosphate pathway and to the TCA Cycle. Among the metabolites of the TCA cycle, the amount of malate, fumarate, succinyl CoA and succinate are increased under photomixotrophic conditions (figure 52). In cyanobacteria lacking a complete TCA cycle (Zhang and Bryant, 2011), such as the marine picocyanobacteria here studied, succinate is one of the terminal metabolites of the TCA cycle and is a precursor of some biosynthetic reactions Besides, it is the substrate for succinate dehydrogenase, that is a major component of the cyanobacterial respiratory electron transport chain (Cooley and Vermaas, 2001).

Previous studies have shown no significant difference in the photosynthetic efficiency of *Prochlorococcus* cultures after addition of low concentrations of glucose (Muñoz-Marín et al, 2017). Future experiments will address whether addition of higher glucose concentrations (such as 5 mM, studied in the present work) might provoke significant changes on photosynthesis in marine picocyanobacteria.

The concentration of sedoheptulose-7-phosphate showed a trend to decrease in all *Prochlorococcus* and *Synechococcus* strains in the presence of glucose (figure 53), contrary to what was reported in *Synechocystis* sp. PCC 6803, in which they were higher under mixotrophic conditions (Yoshikawa et al., 2013). This suggests that this metabolite acts differently in *Synechocystis* sp. PCC 6803 vs the picocyanobacteria studied in this

work. However, sedoheptulose shows a high increment specially in *Prochlorococcus* strains (figure 53). 3-phospoglycerate shows a clear increment in all strains, except in *Prochlorococcus* sp. SS120 (figure 53). Late TCA cycle intermediates were higher in all strains relative to controls or low glucose samples (figure 52), these results are in agreement with those reported by Yoshikawa and coworkers where intracellular levels of TCA cycle metabolites, such as cis-aconitate and succinate, were higher under mixotrophic conditions (Yoshikawa et al., 2013). Decrease of succinate dehydrogenase activity could be the cause of succinate increase under photomixotrophic conditions. Interestingly, the opposite has been reported in *Synechocystis* sp. PCC 6803 where succinate decreased under photomixotrophic conditions (Takahashi et al., 2008). Moreover, it is worth noting that the results of ^{13}C-metabolic flux analysis suggested that the TCA cycle was less active during autotrophic growth (Young et al., 2011). The levels of these TCA cycle metabolites therefore increase under mixotrophic conditions (Yoshikawa et al., 2013)

CONCLUSIONS

CONCLUSIONS

-The changes in *glcH* expression after addition of increasing concentrations of glucose indicates that *Prochlorococcus* and *Synechococcus* detect these concentrations, adapting the expression of the transporter accordingly.

-The darkness-induced decrease of *glcH* expression shows the active nature of glucose uptake in marine picocyanobacteria. This decrease is most pronounced in *Prochlorococcus* MIT9313.

-The results of metabolomics and proteomics confirm that *Prochlorococcus* and *Synechococcus* use glucose.

-5 mM glucose addition led to a strong metabolic shift toward overall anabolic patterns in all the studied cyanobacterial strains.

-The high affinity glucose transporter from *Prochlorococcus* sp. strain SS120 (GlcH) has been overexpressed and purified.

-Two mutants in selected essential amino acid residues of GlcH, Asp52 and Arg134, have been constructed by directed mutagenesis.

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